USING THE TS65DN MOUSE MODEL OF DOWN SYNDROME TO UNDERSTAND THE GENETICS OF CONGENITAL HEART DEFECTS

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

March, 2014

Abstract

Down syndrome (DS) is the most common chromosomal abnormality in humans, caused by having three copies of human chromosome 21 (Hsa21). It is associated with a variety of features affecting almost every organ system, including the heart. There is an especially high incidence of congenital heart defect (CHD) in DS, where 40 – 50% of affected individuals have a CHD. CHD is the most common congenital defect in live births. The fact that half of those with DS have a normal heart suggests that additional genetic and environmental factors interact with trisomy 21 to cause CHD. Thus, people with trisomy 21 are sensitized to CHD.

The Ts65Dn mouse model of DS was used as an analogous sensitized population to study the role of the *Tbx5* gene in CHD. *Tbx5* is a modifier of CHD known to play a role in chamber formation and septation of the heart. A *Tbx5* null allele was introduced to Ts65Dn mice, and newborn pups were sacrificed and examined for CHDs. There is a significant difference between trisomic and euploid pups in the frequency of overriding aorta (OA). About 58% of the trisomic $Tbx5^{+/-}$ mice present with OA and a ventricular septal defect (VSD), while only 18% of the euploid pups have this defect. These results suggest that there is an interaction between *Tbx5* and trisomy to increase the frequency of specific defects, and suggests a role for *Tbx5* in development of the aorta. Results also suggest that the Tbx5 protein is involved in the transcriptional regulation of trisomic genes.

The possibility of using the Ts65Dn mouse model to study the role of the Sonic hedgehog (Shh) pathway in CHD is also discussed here. Shh is known to be involved in heart development; Shh null mouse embryos exhibit a variety of heart defects. Ts65Dn

mice showed a deficit in response to Shh in pharyngeal arches 1 and 2. If this deficit extends to all trisomic cells then it could contribute to CHD in trisomy. Pharyngeal arches 4 and 6 contribute to heart development and a Shh deficit in those regions would certainly contribute to heart defects. Trisomy is a destabilizing factor that, along with other modifiers, can cause a significant insult to heart development. These studies help in understanding the influence of those factors on heart development.

Readers: Dr. Roger H. Reeves, Dr. Valerie DeLeon

Acknowledgments

I would like to thank everyone who has made this achievement possible. I would first like to thank all of my collaborators who were essential to this work. Peter Gergics and Sally Camper were essential to the *in situ* hybridization experiments presented here. Peter performed the experiments after I attempted them multiple times to no avail. Sally was also helpful in the design of the experiment, and I am extremely grateful for her willingness to help. I am also extremely grateful to Dr. Ivan Moskowitz and his lab members for reviewing and confirming the heart defects in every single mouse that I examined.

I would also like to thank the members of my thesis committee, Debbie Andrew, Valerie DeLeon, Andy McCallion, and Jonathan Pevsner for donating their time and effort. Their advice and help was invaluable to the completion of this work. I would especially like to thank Valerie DeLeon for serving as a reader, for her expert instruction in using the AMIRA computer program to analyze the micro-CT based images I received from Numira Biosciences, and for reviewing several difficult to interpret histology slides with me.

I would also like to thank everyone in the Reeves' lab for their advice, help, and friendship, all of which was essential to my work. Of special note is Huiqing Li, a former postdoctoral scholar in the lab who verified many of the histology results presented here. Roger Reeves, my advisor and mentor, deserves much of the credit for my scientific accomplishments. Without his guidance and support I would not have been able to accomplish this feat.

Finally, I would like to thank my family and friends. My parents and sister have provided me with so much support during these last six years. Without their encouragement I definitely would not have made it to this point.

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Chapter 1: Introduction

Down syndrome (DS), a result of having three copies of all or part of chromosome 21, is the most common live born autosomal aneuploidy. It occurs in about 1 in 700 live births [1]. The first clinical descriptions of DS were made by Jean-Étienne Dominique Esquirol and Édouard Séguin in 1838 and 1846, respectively. John Langdon Down provided a fuller clinical description in 1866 [2]. Dr. Jérôme Lejeune identified trisomy of human chromosome 21 (Hsa21) as the cause in 1959 [3]. In 1961 leading doctors and geneticists decided that the common term for the syndrome, "Mongolism," was inappropriate and the name "Down Syndrome" was suggested in honor of Dr. Down. Most cases of trisomy 21 are due to the inheritance of an extra freely segregating Hsa21, but the syndrome can also be caused by a Robertsonian translocation between chromosome 21 and another chromosome in a parent of an individual with DS. Approximately 98% of cases result from errors in meiosis, and around 70% of those cases occur at meiosis I [4]. In prenatally diagnosed trisomy 21 fetuses 89% of the additional chromosomes were of maternal origin, and 11% were of paternal origin [5].

DS is a complex developmental disorder with over 80 clinical features [6]; the most recognized being intellectual disability and craniofacial dysmorphology, occurring in all those diagnosed with DS. It is the most common genetic cause of intellectual disability and a leading cause of congenital heart defects (CHDs) [7]. CHDs are the most common type of birth defect in live births [8], occurring in 1% of the population [9, 10]. CHD is the most common cause of infant mortality due to birth defect and there are currently no ways to prevent it. Furthermore, there is still much to be determined about the genetic origins of CHD.

The heart is the first functional organ in the embryo, indicating its importance to growth and survival. Early in development, presumptive heart cells from the primitive streak migrate bilaterally towards the midline. The bilateral heart fields unite to form the cardiac crescent (or cardiogenic mesoderm) [11]. This fusion creates a single tube at the midline. The posterior and anterior domains of the heart tube are established by a retinoic acid gradient, committing the posterior heart to becoming the atria [12]. The cardiac crescent consist of myocardium and endocardium, and its cells are known as the primary heart field (PHF). A second group of cells, known as the second heart field (SHF), are added to the crescent to help form and expand the tube [13-15]. The anterior-posterior polarity of the tube is converted to a left-right polarity with looping of the tube.

After looping, endocardial cushions form from endothelial cells in the heart tube that undergo epithelial to mesenchymal transition (EMT) and migrate to regions filled with cardiac jelly. Two sets of these cushions form in the heart; one set partition the primitive atria and ventricles and the other set forms in the conotruncal region which will eventually give rise to the aorta and pulmonary artery. The cushions will eventually fuse and develop into parts of the septa and valves of the mature heart. Defects in endocardial cushion formation, also known as atrioventricular septal defects (AVSD), are associated with DS [16].

Heart looping brings about the formation of the different chambers of the heart, and brings the primitive chambers into the positions they will occupy in the final, mature heart. The PHF contributes mostly to the atria and left ventricle of the heart, while the SHF contributes mostly to the outflow tract (OFT) and right ventricle. After heart looping, septation of the chambers begins. The atrial septa are formed by the convergence

of two separate septa, the septum primum and the septum secundum. Atrial septation begins with the formation of the septum primum, which grows from the roof of the posterior atrial wall. The septum primum eventually fuses with the endocardial cushions, while apoptosis in the superior part of the septum primum creates an opening called the foramen secundum. Defects in the formation of the foramen secundum are the most common cause of ASDs [17-19]. The septum secundum then grows to fuse with the endocardial cushions, leaving an opening called the foramen ovale. At or shortly after birth, pressure in the left atrium forces the septum primum against the foramen ovale, closing the opening and allowing for proper circulation.

Septation of the OFT occurs at the same time as ventricular septation. Prior to its septation the OFT begins to rotate, while the endocardium undergoes EMT and endocardial cushions form in the OFT. The cushions fuse and, along with the addition of cells that have migrated from the neural crest, separate the OFT. The OFT gives rise to the aorta, which connects to the left ventricle, and the pulmonary artery, which connects to the right ventricle. The ventricular septum is formed from two parts: the muscular and membranous portions. The muscular septum grows upwards towards the endocardial cushions, but stops growing before full septation is complete. Cells from both the left and right sides of the primitive ventricle form the septum but do not mix. The membranous portion is formed later, from the fusing of the encodardial cushions of the OFT. The septum is complete upon the joining of the endocardial cushions, atrial septum, muscular ventricular septum, and the dorsal mesenchymal protrusion (DMP).

There are a variety of genes and molecular pathways involved in heart development, adding to its complexity. Genes that regulate development control various

pathways such as signal transduction, chromatin regulation, transcription, and extracellular matrix production (reviewed in [20]). The Notch, Sonic hedgehog (Shh) and NFAT signaling pathways are important for the EMT process in the endocardial cushions of the heart. Defects in these signal transduction pathways can lead to defective development of the endocardial cushions and septation errors. Mutations in genes that encode for transcription factors can also lead to various heart defects. The T-box and GATA families of transcription factors are important for heart development. Pitx2, another transcription factor, plays an important role in the left-right asymmetry of the heart.

Chromatin regulators and histone modifiers influence heart development as well. Mutations in the chromatin regulator Brg1, cause ventricular septal defects (VSDs) and problems in OFT development [21, 22]. Muations in histone deacetylases (HDACs) can also cause errors in development. Mouse knockouts of *Hdac3* show VSDs and defects in the alignment of the aorta [23]. Even mutations in extracellular matrix (ECM) proteins can lead to heart defects. Mutations in the ECM protein Fbln1 cause a variety of defects in the heart and affect septation and alignment of the OFT [24]. Mutations in the cell adhesion protein Ptk2 can cause defects affecting every structure in the heart (reviewed in [20]). Adding to its complexity, mutations in non-coding microRNAs have been found to lead to CHD as well. Mutations in *mir17*, *mir1a-2*, and *mir133a* have all been associated with VSDs (reviewed in[20]). From the observation of the many pathways involved in heart development, it is clear that heart development is a highly complex process and one that requires the interaction of many different pathways, many of which may still be unknown.

The Down Syndrome Heart Project (DSHP) aims to gather genetic information from those with DS and CHD and to use that information to study the genetic origins of CHD. Forty to fifty percent of individuals with DS have some type of CHD. These are mostly septal defects of the heart, with AVSD being the most common. Of those individuals with DS and a CHD, approximately 20% have an AVSD. The frequency of AVSD in DS is about 2000 times higher than in the general population [25]. Since half of those with DS have a normal heart, additional genetic and environmental factors must interact with DS to cause CHD, i.e., trisomy 21 sensitizes the fetus to CHD, but is not sufficient to cause it. Thus, dosage effects of Hsa21 genes are a complex modifier that, in conjunction with other risk factors like single-gene variants, may increase susceptibility to CHD (Figure 1). Attempts to identify genes predisposing to CHD in DS have understandably focused on Hsa21, while there has been little consideration of disomic modifiers that contribute to this increased risk. The DSHP uses the sensitized DS population to find disomic genetic modifiers of CHD that may contribute to CHD in all people, not just those with DS.

Animal models are an important tool for understanding the pathogenesis of CHD and the molecular mechanisms that give rise to these conditions. Mice are small and easily housed and they exist as inbred strains, minimizing the effects of genetic variability. Heart development in the mouse is analogous to development in humans, making it a prime model system to study heart development. Conserved synteny between the mouse and human also make the animals ideal for study. Orthologs of many genes on

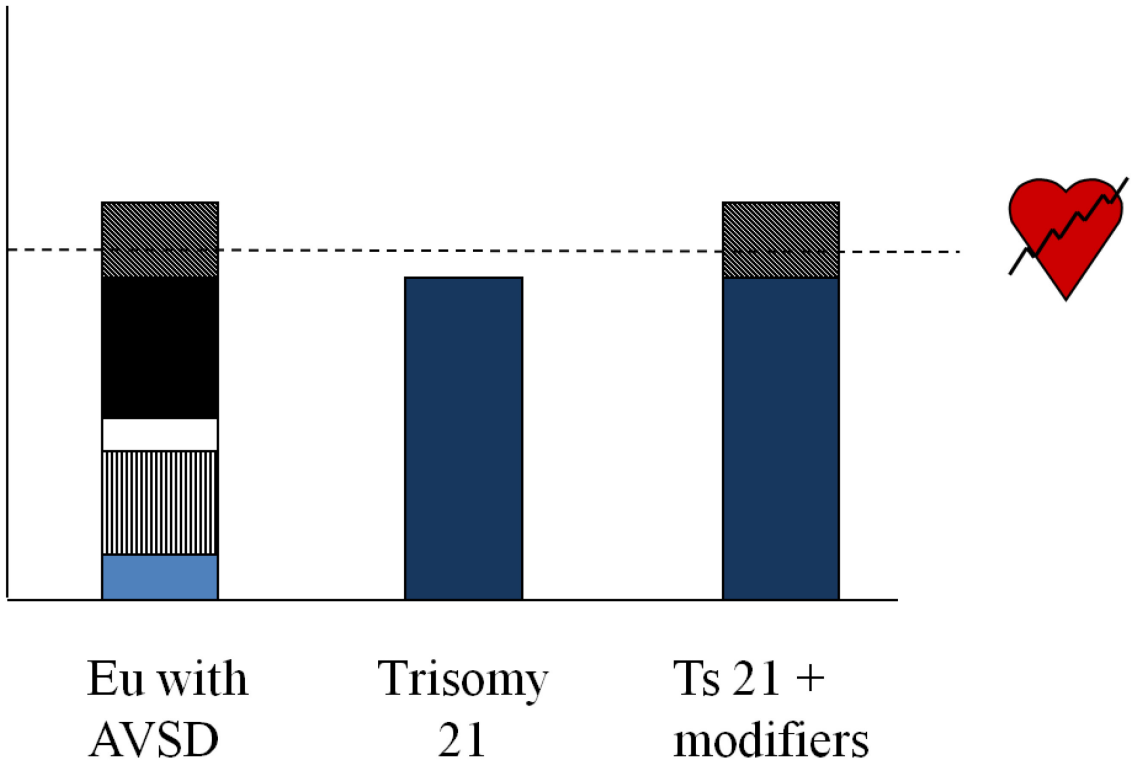


Figure 1. Threshold Hypothesis. Additive effects of individual modifier genes can reach a threshold whereby heart septal development is disrupted, but the likelihood of inheriting many predisposing modifiers is small (Eu with AVSD). Trisomy 21 is a significant risk factor for CHD, but does not cause it (Trisomy 21). People with an extra copy of Hsa21 may require fewer disomic or trisomic modifiers to reach the threshold (Ts 21 + modifiers). The relative contribution of the modifier in the sensitized Down syndrome population is therefore more readily detectable. Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: Li, H., *et al.* CIRCULATION: CARDIOVASCULAR GENETICS 5 (3):301-308, copyright (2012).

Hsa21 are found on mouse chromosome 16 (Mmu16), with smaller subsets on Mmu10 and Mmu17 (Figure 2) [26]. Several mouse models have been generated to replicate the symptoms of DS in mice (Figure 3). The Ts1Cje, Ts1Rhr, and Ts65Dn mouse models are all partial trisomies of Mmu16, replicating various clinical features of DS [27-29]. The Tc1 mouse is a so-called "transchromosomic" line, carrying an almost complete copy of Hsa 21 [30]. In recent years two new mouse models of DS have been developed. The Dp(16)1Yey/+ mouse is trisomic for all regions of Mmu16 that are orthologous to Hsa21 [31], and the Dp(16)1Yey/+, Dp(17)1Yey/+, Dp(10)1Yey/+ mouse is trisomic for all regions of the mouse genome orthologous to Hsa21 [32]. The most widely studied DS mouse model, Ts65Dn, is trisomic for a segment of Mmu16 containing about half of the mouse genes orthologous to Hsa21 [33]. These mice were the result of an induced chromosome breakage and translocation between Mmu17 and Mmu16. Ts65Dn mice display a number of the features of DS, but not all. Ts65Dn mice have a similar craniofacial dysmorphology to that seen in DS [34], and also display a smaller cerebellum, analogous to what is seen in DS [35]. Ts65Dn mice do exhibit cardiac abnormalities, although these occur at a lower frequency than in humans [36, 37]. Four percent of Ts65Dn mice have a septal defect, while 18% have OFT defects [36].

The goal of this thesis was to expand knowledge of the genetic pathways involved in the origin of CHD. Using the Ts65Dn mouse model we set out to examine a disomic modifier of CHD, the *Tbx5* gene, and investigate the genetic origin of CHD. Trisomic populations are sensitized to CHD, making them prime candidates for this investigation. A multitude of genes affecting diverse pathways affect heart development, indicating that an interaction between trisomic and disomic genes during development is likely. We

aimed to delve into those interactions by focusing on a known modifier of heart development, *Tbx5*, and its interaction with trisomy in the Ts65Dn mouse model.

Figure 2. Hsa21 orthologs on Mmu10, 16, and 17. Hsa21 is syntenic with regions on Mmu 17, Mmu 16, and Mmu 10. The largest region of synteny is on Mmu16. Reprinted by permission from Macmillan Publishers Ltd: Antonarakis,S.E. *et al*. NATURE REVIEWS GENETICS 5(10): 725-738, copyright (2004).

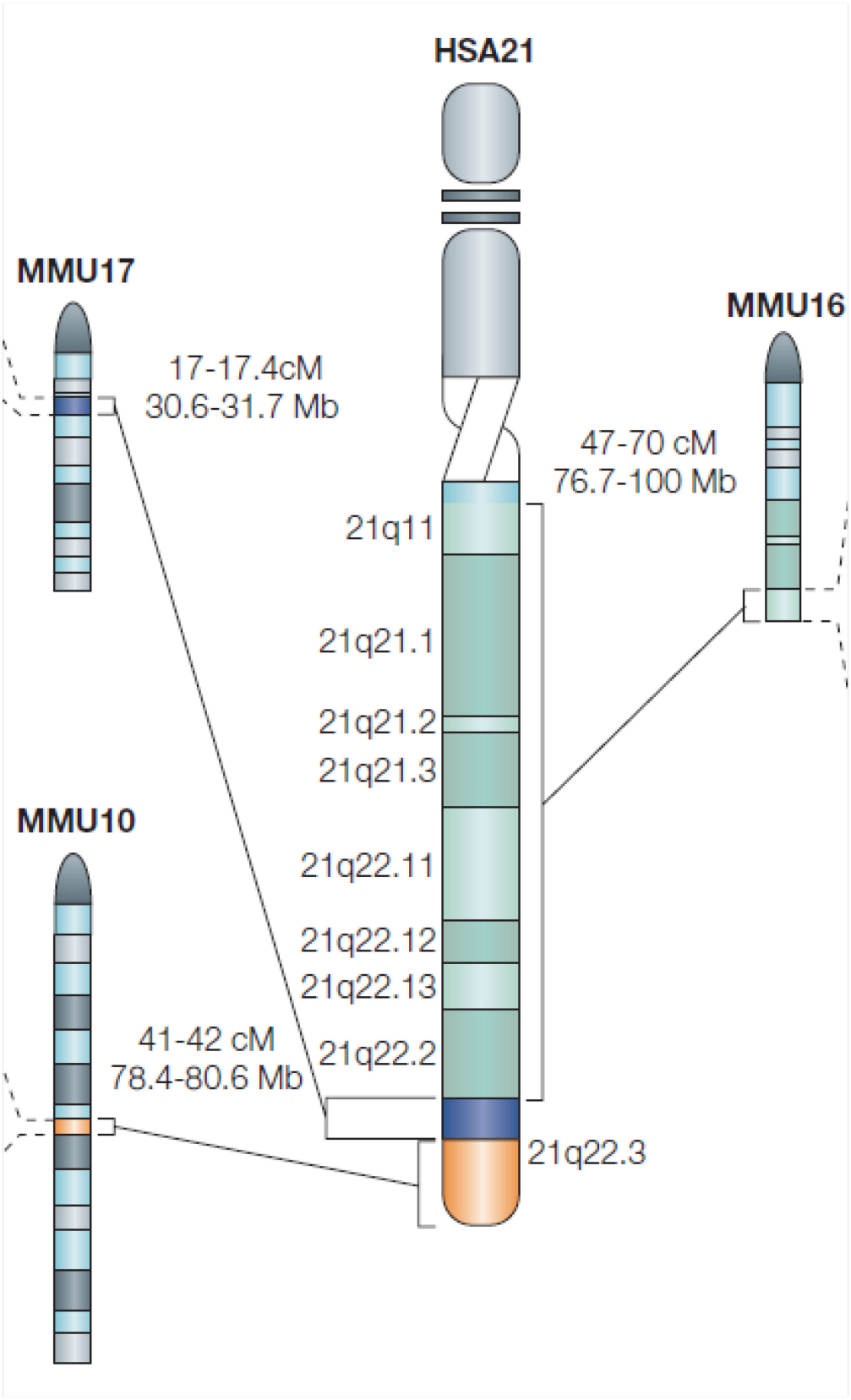
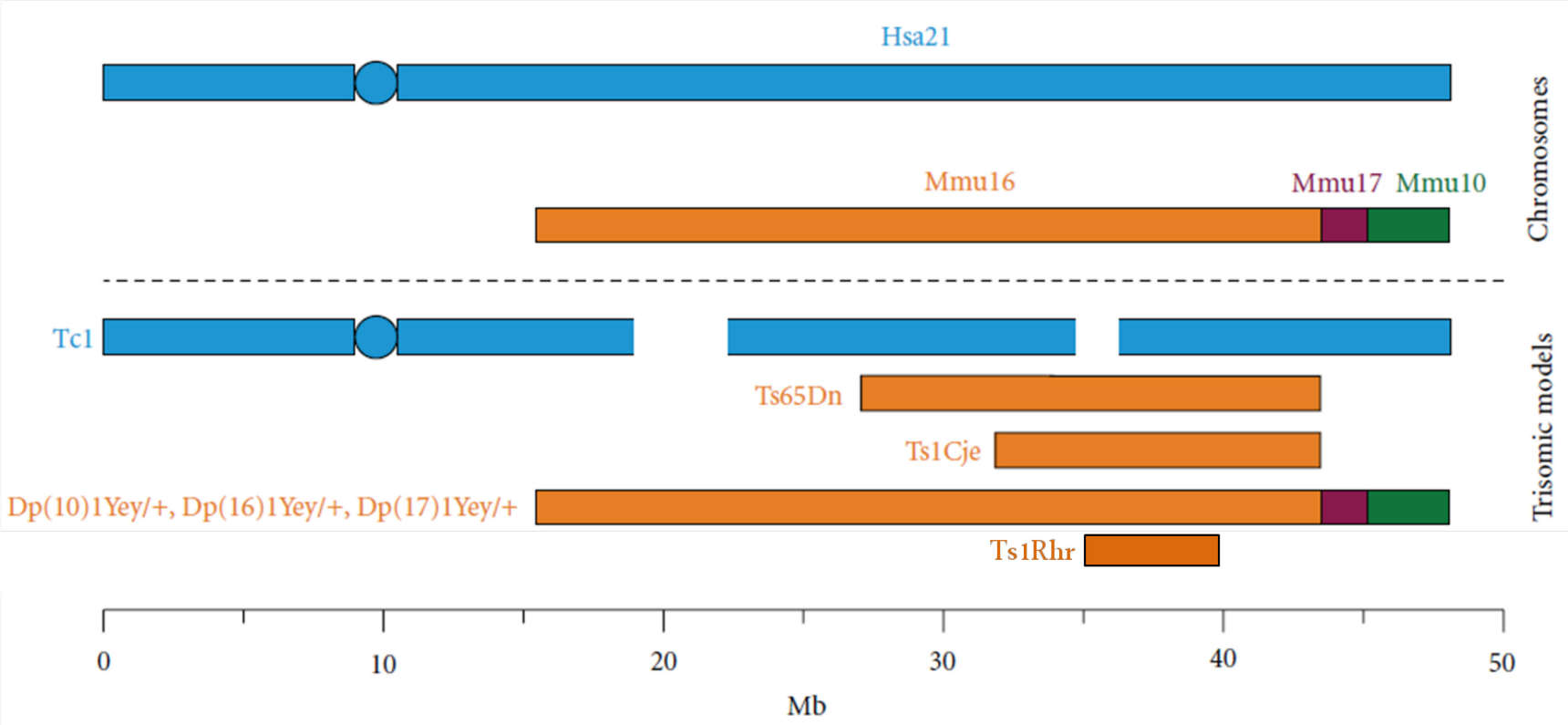

HSA21
MMU17
MMU16
MMU10
17-17.4cM
30.6-31.7 Mb
47-70 cM
76.7-100 Mb
41-42 cM
78.4-80.6 Mb
21q11
21q21.1
21q21.2
21q21.3
21q22.11
21q22.12
21q22.13
21q22.2
21q22.3

Figure 3. Mouse models of DS. Hsa21 (in blue) and the syntenic mouse chromosomes (Mmu 16, orange, Mmu 17, purple, Mmu10, green). The trisomic regions of several of the well-established mouse models of DS are aligned to the corresponding parts of the human and mouse genome. Adapted from Sheppard, O. *et al.* Mouse models of aneuploidy. *The Scientific World Journal.* 2012; 2012:214078. Reprinted with permission under the Creative Commons Attribution License.


Hsa21
Mmu16
Mmu17
Mmu10
Chromosomes
Tc1
Ts65Dn
Ts1Cje
Dp(10)1Yey/+, Dp(16)1Yey/+, Dp(17)1Yey/+
Ts1Rhr
Trisomic models
0
10
20
30
40
50
Mb

Chapter 2: *Tbx5* and trisomy interact to alter the incidence of specific heart defects

Introduction

We consider a known contributor to heart development, *Tbx5*, and its role as a disomic modifier of septal defects in Ts65Dn mice. *Tbx5* is a member of the T-box family of transcription factors. The defining family feature is a conserved sequence in a DNA binding domain that extends across a region of 180-200 amino acid residues called the T domain [38, 39]. All members of the family are known to be involved in regulation of transcriptional activity, some as activators and others as repressors. *Tbx5* activation of transcription has well-described effects in cardiac and limb development [40, 41]. Mutations in the gene are associated with Holt-Oram syndrome; patients diagnosed with the syndrome present with various heart defects and skeletal abnormalities in the hands and arms. About 85% of affected individuals have a structural heart defect and/or abnormalities in the cardiac conduction system. Holt-Oram patients most often present with ASDs and VSDs.

Several different roles for the gene in cardiogenesis have been found. *Tbx5* has been found to play a role in activating transcription of various target genes including *Gja,* and *Anf* [42]. In order to carry out its transcriptional activity the protein has been found to interact with various other transcription factors including Sal4 [43], Mef2c [44], myocardin [45], and members of the GATA family [46, 47]. *Tbx5* is important for cardiomyocyte differentiation [48, 49], cell proliferation [50, 51], and cardiac conduction [52]. Transcription of the gene is also required for reprogramming differentiated somatic cells into functional cardiomyocyte-like cells [53]. The majority of work regarding *Tbx5* has focused on its essential role in formation of the heart chambers. The gene is

expressed in the left ventricle (LV) and both atria during chamber maturation and septation [54]. The ventricular septum forms at the boundary between Tbx5-expressing LV and non Tbx5-expressing RV [54, 55]. Ectopic expression in the right ventricle or deletion of *Tbx5* in the left ventricle of mice suppresses formation of the ventricular septum, resulting in formation of a single ventricle [56]. Conditional knockouts of the gene in the SHF cause ASDs to form in mice [57]. Its importance in septation of the heart suggests that variants affecting *Tbx5* expression might affect septation in a trisomic (Ts) mouse model of DS. We examined the role of *Tbx5* in septal development by crossing Ts65Dn mice with mice heterozygous for a null allele for *Tbx5*.

We used a mouse model with a null allele for *Tbx5* to study the effects of this gene on septal development in conjunction with trisomy [42]. Homozygous null $Tbx5^{-/-}$ embryos (Black Swiss/SvJ background) die by embryonic day 10.5 (E10.5) and lack cardiac looping and endocardial cushion formation, among other severe defects [42]. Heterozygote mice on that same genetic background exhibit ASDs and VSDs. The viability of and defects observed in $Tbx5^{+/-}$ mice is greatly influenced by genetic background. Deviation from the expected Mendelian frequency varies with the genetic background of the mice, indicating that prenatal loss has occurred and that difference in genetic background plays a role in phenotype. Thus, the effects of *Tbx5* dosage are susceptible to additional genetic modifiers.

The interaction between *Tbx5* dosage and trisomy showed a dramatic increase in defects affecting aortic alignment and an effect on left-right patterning of the heart. The *Pitx2* gene is an important determinant of left-right asymmetry of the heart. It confers left atrial identity and when it is absent or ectopically expressed, atrial isomerism results [58-

60]. Other transcription factors in the T-box family are known to regulate *Pitx2* expression [61], and *Pitx2* regulates *Tbx5* expression in the abdominal wall during development [62]. *Pitx2* expression in Trisomic (Ts), $Tbx5^{+/-}$ embryos was examined to look for altered atrial identity in these mice. The molecular mechanisms by which *Tbx5* influences heart development are incompletely described and its possible interactions with genes on Hsa21 are unknown. Here we provide evidence of an interaction between *Tbx5* and trisomy, and the effects of that interaction on trisomic gene expression and left-right patterning of the heart.

Methods

Animal Husbandry

Animals were maintained in a virus and antibody-free facility with food and water *ad libitum*. Ts65Dn mice (B6EiC3H-a/A-Ts(17^{16})65Dn) were obtained from The Jackson Laboratory and maintained on the B6;C3H background. *Tbx5* heterozygous null mice ($Tbx5^{+/-}$) were obtained from Dr. Jonathan Seidman [42]. We backcrossed the $Tbx5^{+/-}$ mice onto a C57BL/6J background. Mice used in this study were the progeny of female Ts65Dn mice. All procedures were approved by the Institutional Animal Care and Use Committee.

Genotyping

Genomic DNA was extracted from tail tips of mice by heating at 90°C for 2 hours in 10mM NaOH, 0.2mM EDTA, and used for genotyping by PCR. Sequences of primers for Ts65Dn genotyping are as follows: C17F: 5'-GTGGCAAGAGACTCAAATTCAAC-3'; C16R: 5'-TGGCTTATTATTATCAGGGCATTT-3'; IMR5: 5'-

AAAGTCGCTCTGAGTTGTTAT-3'; IMR6: 5'-GGAGCGGGAGAAATGGATATG-3'. For Ts65Dn genotyping, PCR was done under the following cycling conditions: 95°C 3 min, (94 ˚C 10s, 58.7˚C 20s, 72˚C 27s) for 31 cycles, 72 ˚C 5 min. In the cuploid mice a region of 600 base pairs (bp) was amplified. This band in addition to 275 bp band was seen in trisomic mice. For *Tbx5* genotyping, three primers designed to amplify either the wild type or null alleles were added together in each reaction [42]. The sequences of the primers for *Tbx5* genotyping are as follows: 3F2: 5'-CCCAGCGGCAGGCGTAGAC -3'; Loxp-F: 5'-GCAGCGCAGTCCTCACCAG -3'; Loxp-R: 5'-AAATTCCAACCCCTTCCACAGAT -3'. The PCR was done under the following cycling conditions: 94˚C 3 min, (94 ˚C 30s, 59.7˚C 30s, 72˚C 1 min) for 35 cycles, 72 ˚C 10 min. The region amplified in the mutant allele was 480 bp, and the region amplified in the wild type allele was 158 bp.

Histology

All progeny of Ts65Dn x $Tbx5^{+/-}$ crosses were collected at postnatal day 0 (P0) within hours of birth. The pups were euthanized and thoraxes were removed and fixed in 10% formalin for at least 48 hours. The tissue was dehydrated in 30%, 50%, and 70% ethanol for 1 hour each before being stored at 4°C until embedded in paraffin. The paraffin blocks were sectioned at 7μm by standard methods, and stained with hematoxylin/eosin using standard methods. The heart morphology in each animal was scored under a dissecting stereomicroscope (Nikon SMZ1500, Japan) by at least two individuals blinded to genotype. Dr. Ivan Moskowitz also verified all CHDs. Pictures were taken using the NIS-Elements Br software (Nikon).

In Situ Hybridization

E13.5 embryos were fixed in 4% paraformaldehyde for 1 hour, rinsed in 1X PBS three times for 5 minutes, and then dehydrated in 25%, 50%, and 70% ethanol for 1 hour each. After dehydration the embryos were stored at -20°C in 70% ethanol until embedded in paraffin and sectioned according to standard methods. We performed in situ hybridization using traditional dioxigenin (DIG)-labeled RNA probes detected with alkaline phosphatase (AP)-conjugated anti-dioxigenin antibody using nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl-phosphate toluidine-salt (NBT/BCIP) substrate. DIG RNA labeling mix, anti-DIG-AP antibody, blocking reagent, NBT/BCIP were all purchased from Roche Applied Science (Indianapolis, IN) and used according to the manufacturer's instructions with minimal modifications. The Pitx2 antisense and sense probes were described earlier [63]. Images were taken with the Leica DMRB upright light microscope using the Leica Application Suite Software v4.3 (Leica Microsystems-W. Nuhsbaum Inc., McHenry, IL). Composite images were compiled using Adobe Illustrator CS4 (Adobe Systems, San Jose, CA).

Statistical Analysis

A chi-squared test was used to determine if the occurrence of genotypes resulting from a Ts65Dn x $Tbx5^{+/-}$ mating fit Mendelian ratios. The incidence of various heart defects for different genotypes was compared by Fisher's test using GraphPad Prism version 5. A 2x2 contingency table was constructed for each defect, comparing animals that had that particular defect and animals that did not. A p-value where $p<0.05$ was considered significant.

Results

Viability of Tbx5$^{+/-}$ mice is dependent on genetic background

B6.*Tbx5*$^{+/-}$ male mice were crossed to Ts65Dn females to examine the role of *Tbx5* as a modifier of CHD. Genetic background of the *Tbx5* mice affected viability (Table 1). At birth, *Tbx5* genotypes appeared at Mendelian ratios (χ^2=1.42, df=1, p=0.23) on a B6 x C3H (75% B6, 25% C3H) background (Table 2), but by weaning, the frequency of the *Tbx5*$^{+/-}$ genotype was 21% rather than the expected 50%. On an inbred B6 background, the frequency of the *Tbx5*$^{+/-}$ genotype is 9% at weaning. Thus, it appears that genetic factors contribute to embryonic and perinatal lethality associated with *Tbx5*$^{+/-}$.

Trisomy affects patterns of CHD in Tbx5$^{+/-}$ mice

Progeny of the Ts65Dn x *Tbx5*$^{+/-}$ crosses were collected within hours of birth, prepared for histology, and assessed for the presence of CHD. All animals examined for CHD were on the B6 x C3H (75% B6, 25%C3H) background. There is some loss of Ts65Dn fetuses during late gestation, and the observed frequency of 42% trisomic pups at P0 (Table 2) was in the expected range [64]. CHD is highly penetrant in *Tbx5*$^{+/-}$ mice and we saw only a slight overall increase in the percentage of heart defects when the null allele occurred on a trisomic background (Table 3). However, the pattern of effects was altered significantly by trisomy.

We observed overriding aorta (OA) in ~58% of Ts, *Tbx5*$^{+/-}$ mice but only ~18% of euploid (Eu), *Tbx5*$^{+/-}$, a significant difference (Table 3, p=0.0004). OA consists of a VSD and an improperly positioned aorta directly over the VSD (Figure 4). A difference in penetrance of AVSD, with 5% of *Tbx5*$^{+/-}$ mice exhibiting an AVSD and ~19% of their

Table 1. Viability of *Tbx5*$^{+/-}$ mice is influenced by genetic background

Genetic Background	Frequency of *Tbx5*$^{+/-}$ genotype at weaning
C57BL/6J	9% (n= 528)
B6;C3H (75% B6, 25% C3H)	21% (n=131)
129SvEv/129SvJ	10%[a]
Black Swiss/SvJ	28%[a]

[a]*Bruneau, B.G., et al., A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell, 2001. 106(6): p. 709-21.*

Table 2. Ratios of all genotypes in a Ts65Dn x *Tbx5*$^{+/-}$ cross on a B6;C3H (75% B6, 25% C3H) background (at birth).

Genotype	Number (%), n=180
Tbx5$^{+/+}$	52 (28.9%)
Tbx5$^{+/-}$	51 (28.3%)
Ts65Dn	46 (25.6%)
Ts65Dn, *Tbx5*$^{+/-}$	31 (17.2%)

Table 3. Congenital heart defects in P0 trisomic and euploid *Tbx5*$^{+/-}$ mice

Genotype	ASD	VSD only	Overriding aorta [b]	Gerbode's Defect	AVSD[c]	No Defect	Total Mice
Euploid *Tbx5*$^{+/-}$	11 (27.5%)	10 (25%)	7 (17.5%)	4 (10%)	2 (5%)	13 (32.5%)	40
Ts65Dn *Tbx5*$^{+/-}$	12 (38.7%)	6 (19.4%)	18 (58.1%)	6 (19.4%)	6 (19.4%)	5 (16%)	31

[a] *Several animals had more than one defect.*

[b] *There is a significant difference between euploid and trisomic Tbx5 heterozygous mice in the occurrence of overriding aorta (p=0.0004).*

[c] *The difference between euploid and trisomic Tbx5 heterozygous mice in AVSD is not significant but a larger sample size may have shown significance. (p=0.07)*

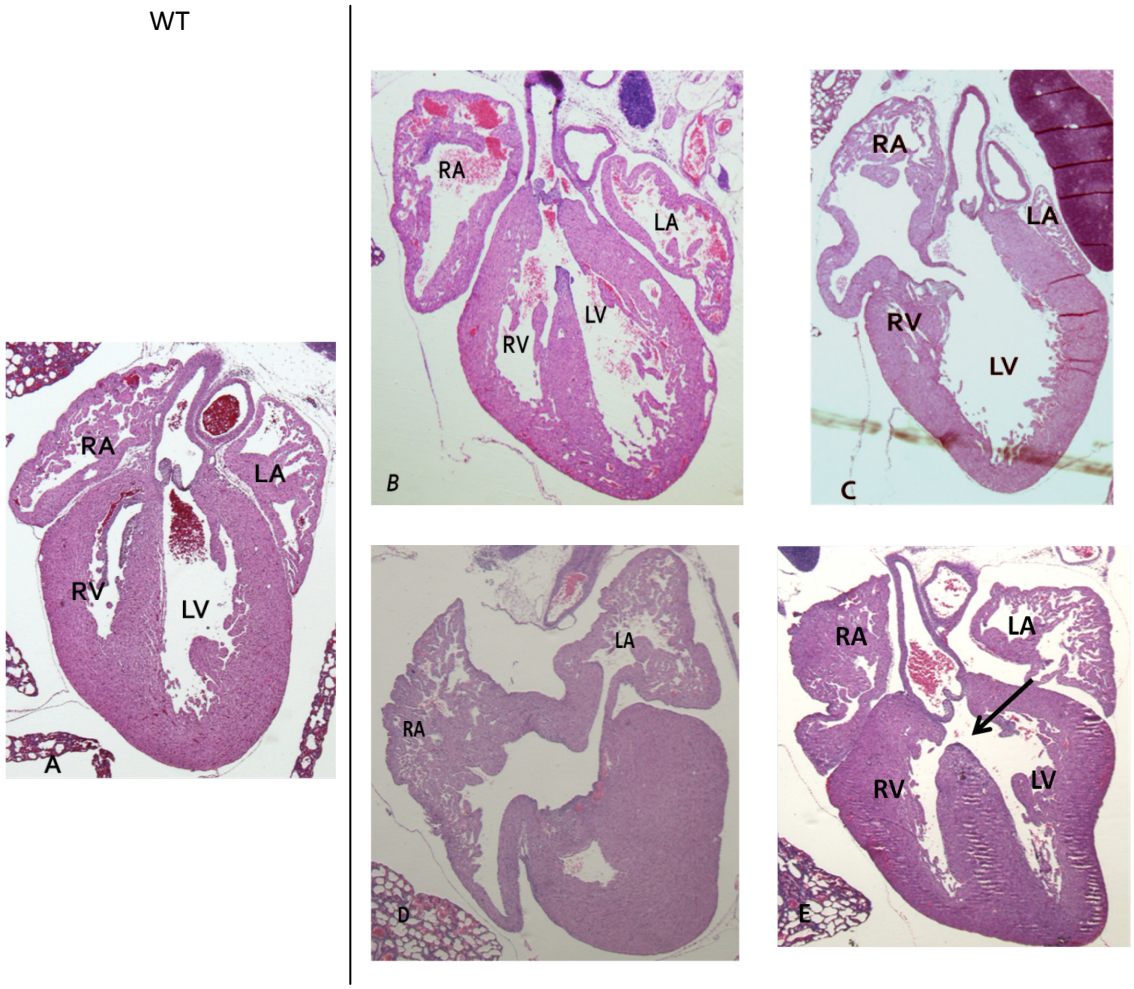


Figure 4. CHDs seen in P0 trisomic and euploid *Tbx5*$^{+/-}$ mice. Coronal histological sections of wild type (A) and Ts, Tbx5$^{+/-}$ (B-D) newborn mice stained with H&E. Overriding aorta (B), Gerbode's defect (C), ASDs (D), and VSDs (E) were seen in the examined animals.

Ts, *Tbx5*$^{+/-}$ littermates exhibiting the defect (p=0.07), were also observed. ASDs, VSDs and Gerbode's defect, an abnormal communication between the right atrium and left ventricle, were also seen (Figure 4) but none reached statistical significance. Numira Biosciences performed Virtual Histology™ on six Ts *Tbx5*$^{+/-}$ mice. Three of these animals were affected, with an AVSD, ASD, and VSD, respectively.

Atrial isomerism in trisomic Tbx5 heterozygotes

OA and Gerbode's defect are seen more often in trisomic *Tbx5*$^{+/-}$ mice than in their *Tbx5*$^{+/-}$ littermates. We hypothesized that these defects might be accompanied by atrial isomerism. In this situation both atria are mirror images of one another and lack the typical morphological and molecular characteristics of the right or left atrium. *Pitx2* is specifically expressed in the left atrium, and its expression is absent or ectopically expressed in the right atrium in cases of atrial isomerism [58]. To determine whether atrial isomerism occurred in trisomic mice, *in situ* hybridization for *Pitx2* was performed on coronal paraffin sections of E13.5 torsos. *Pitx2* expression can be seen in the left atrium of the heart in euploid embryos, but that expression is much weaker in the mutants (Figure 5). The weak *Pitx2* expression in the atria of Ts, *Tbx5*$^{+/-}$ embryos suggests that these embryos have defects in laterality (i.e., atrial isomerism). Atrial isomerism likely contributes to the dramatic increase in OA in the trisomic, *Tbx5*$^{+/-}$ animals. Note that *Pitx2* expression in other organs was comparable between the two genotypes (Figure 6).

Discussion

Heart development is a complex process. The role of genetic modifiers is clear from the differences in penetrance and patterns of heart phenotypes in DS and DS mouse

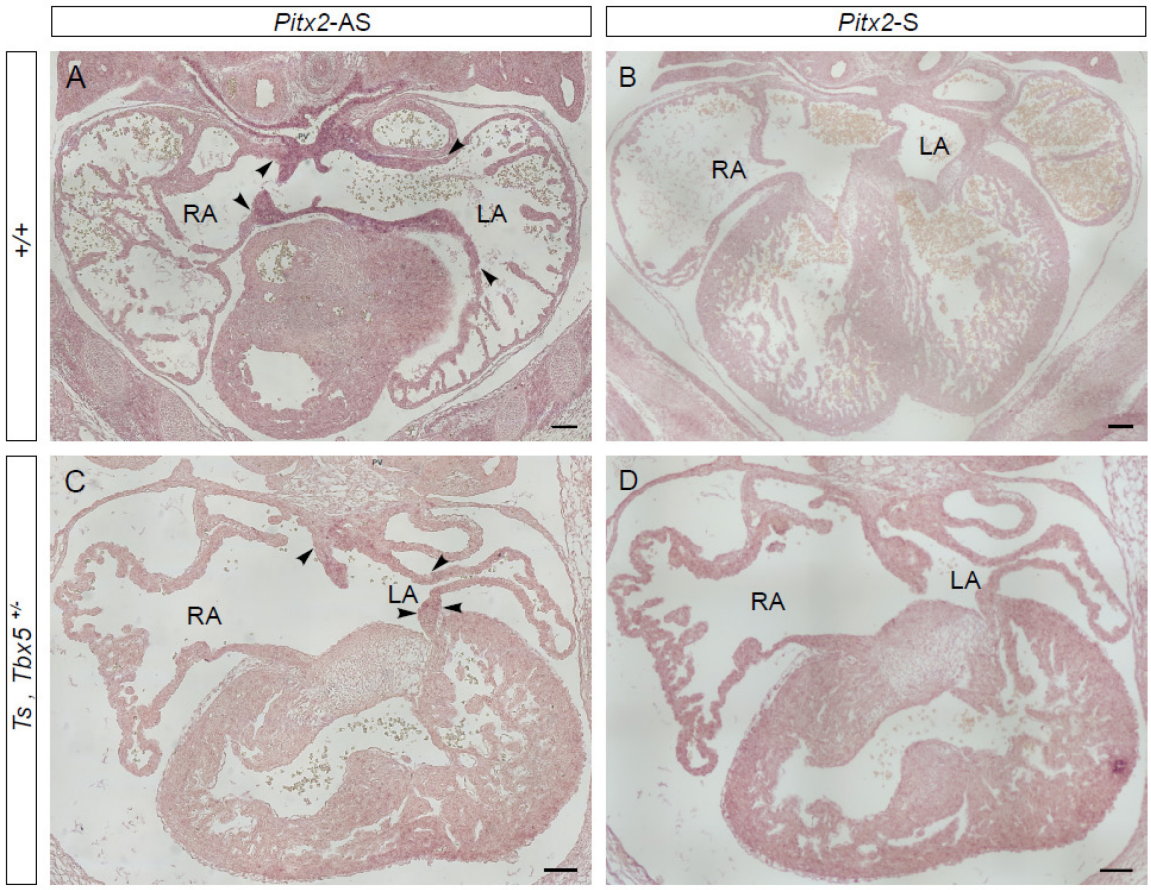


Figure 5. Pitx2 expression in E13.5 hearts to determine atrial isomerism. *In situ* hybridization of coronal sections of wild type (A,B) and Ts, $Tbx5^{+/-}$ (C,D) E13.5 embryonic hearts. Pitx2 expression can be seen in the left atrium of the WT animals (A). Pitx2 expression is much weaker and confined to a smaller area (arrowheads) in the trisomic, $Tbx5^{+/-}$ mutants (C).

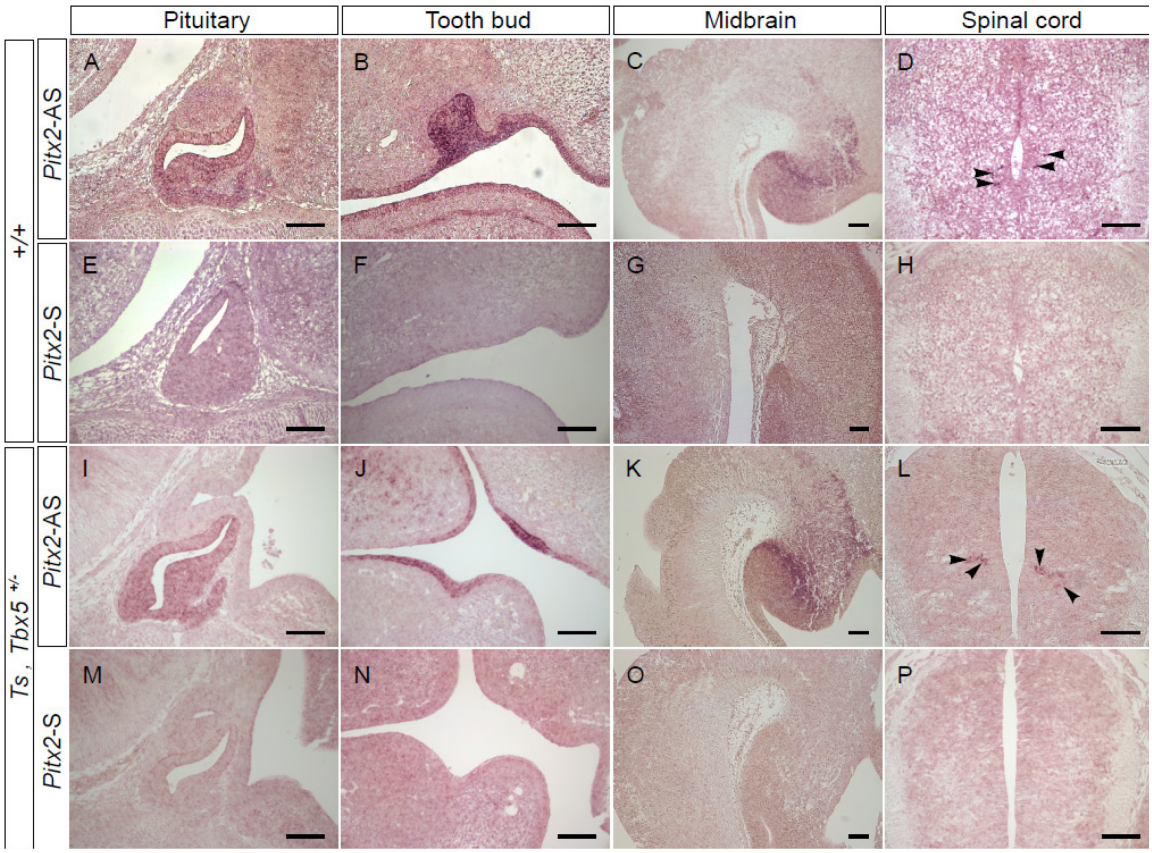


Figure 6**.** *Pitx2* expression in various organs in E13.5 wild type and trisomic, $Tbx5^{+/-}$ mutant embryos. Note that *Pitx2* expression in the trisomic, $Tbx5^{+/-}$ animals is comparable to that seen in wild type embryos in the pituitary, tooth bud, midbrain, and spinal cord. Pitx2-AS: Pitx2 antisense probe, Pitx2-S: Pitx2 sense probe. Bar = 100 µm.

models. Modifiers also affect *Tbx5*, which is seen when the *Tbx5* null allele is bred onto different genetic backgrounds. Holt-Oram patients who inherit the same mutation in *TBX5* have variable phenotypes, indicating that additional factors affect development [65]. Incomplete penetrance of CHD in DS indicates that other interactions between trisomy and other factors can alter developmental outcomes. Previous work from our lab and others has shown effects of the interactions between trisomy and disomic modifier genes, both in both humans and mouse models [25, 37]. Our data support findings in people showing that DS and *TBX5* mutations can cause multiple, severe defects when combined [66]. Individuals with DS and mutations in *TBX5* displayed OA and a number of additional defects.

Previous studies on *Tbx5* and its role in heart development have emphasized its importance to septation of the heart. Because of this we expected to see a difference in susceptibility to septal defects in the trisomic, $Tbx5^{+/-}$ mice. Our results showed no formal statistical significance between the euploid and trisomic mice when looking at ASDs, VSDs, or AVSDs. However, there was a trend towards more AVSD ($p=0.07$) in the trisomic $Tbx5^{+/-}$ mice. While the occurrence of AVSD was not statistically significant, it is possible that a larger sample size may yield significant results. AVSDs are extremely severe and may lead to death *in utero*. It is possible that the trisomic mice show significantly more of these defects, but the mice die prenatally. To answer this question, a future direction would be to repeat this study and look for these defects before birth.

Our data have also demonstrated a role for the *Tbx5* gene beyond cardiac septation. The appearance of OA, a phenotype affecting OFT development, suggests roles for the gene beyond septation. Localization studies have never found *Tbx5* expression in

the OFT in mice, but expression of the gene has been found in the OFT of chick embryos during development [67]. Haploinsufficiency of Tbx5 has been found to hinder cardiomyocyte development, which is known to cause alignment defects of the aorta. Combined, these data suggest that there is a potential role for *Tbx5* in the development of the aorta.

These results show a clear interaction between trisomy and Tbx5 dosage. A combination of the two factors correlated with changes in the expression of the *Pitx2* gene. *Pitx2* regulates left atrial identity, and its expression is critical for development of left-right asymmetry in the heart and gut [58, 59, 68-71]. Defects in atrial identity, or atrial isomerism, occur in mice with reduced *Pitx2* expression [59]. In *Pitx2* mouse mutants the OFT is shifted dorsally towards the right, a condition that contributes to OA [72]. In fact, OA is thought to be a less severe form of a defect called double outlet right ventricle (DORV), in which the aorta arises mostly from the right ventricle instead of the left. *Pitx2* mouse mutants frequently exhibit this defect [60, 69, 71, 73]. Based on these previous reports and our findings that *Pitx2* expression is reduced in the atria of E13.5 Ts, $Tbx5^{+/-}$ embryos, it is likely that atrial identity is altered in these mice, leading to an increase in OA. We have provided evidence suggesting that left-right asymmetry is adversely affected in Ts, $Tbx5^{+/-}$ mice.

We have shown that multiple pathways are affected when trisomy and *Tbx5* haploinsuffiency are combined. The outcome of development can be altered when mutations in a disomic modifier of CHD is combined with trisomy. Alterations in *Pitx2* expression are known to lead to deficits in the left-right pathway and the development of various heart defects. Trisomy and *Tbx5* haploinsufficiency cause a reduction in *Pitx2*

expression, likely causing defects in the left-right asymmetry of the heart in these mice. Our findings document previously unknown connections between Tbx5 and trisomic genes and their influence on the appearance of structural heart defects and left-right patterning when combined.

Table 4. Euploid and trisomic P0 animals with multiple defects

Genotype	No Defect	1 defect	>1 defect	Total Mice
Euploid *Tbx5*$^{+/-}$	13 (32.5%)	13 (32.5%)	14 (35%)	40
Ts65Dn *Tbx5*$^{+/-}$	5 (16.1%)	6 (19.4%)	20 (64.5%)	31

Chapter 3: Trisomic gene interaction with Tbx5

Introduction

Previous studies in our lab have found interactions between trisomy and disomic genes, *Hey2* and *Creld1*. *Hey2* is a member of the hairy and enhancer of split-related (HESR) family of transcription factors. These factors interact with histone deacetylase complexes to repress transcription. Mutations in the gene have been identified in DS patients with CHD but not in euploid individuals with CHD [74, 75]. Homozygous *Hey2* knockout mice die in early embryogenesis and always present with a septal defect [76, 77]. *Hey2* is a disomic modifier of CHD on a trisomic background. $Hey2^{+/-}$ mice presented with no septal defects but the incidence increased to 24% when $Hey2^{+/-}$ mice were put on a triomic background [37].

Creld1 encodes a member of a family of epidermal growth factor related proteins. It was originally identified as a candidate for the AVSD2 locus [78]. The gene is associated with AVSD [16, 79] and mutations in it have been found in people with DS and CHD [37, 80]. $Creld1^{+/-}$ mice did not show any septal defects but the incidence of septal defects increased to 33.3% when the mice were crossed on to a trisomic background [37]. To identify the trisomic gene with which *Creld1* might be interacting, $Creld1^{+/-}$ mice were crossed with the Ts1Rhr and Ts1Cje mouse models of DS. The frequency of septal defects on the Ts1Rhr and Ts1Cje models was unchanged when the *Creld1* haploinsufficiency was added (H. Li, unpublished data). This indicated that a gene trisomic in the Ts65Dn model but not the others was a candidate for that interaction. The *Jam2* gene was chosen as a candidate due to its expression in heart and its implication in CHD in zebrafish (H. Li, unpublished data) and angiogenesis in the Tc1

mouse model of DS [81]. The histology data presented in chapter 2 indicate an interaction between a disomic gene (*Tbx5)* and trisomy. Here, the Ts65Dn mouse model was used to identify trisomic genes that interact with *Tbx5*.

The molecular mechanisms by which *Tbx5* influences heart development are incompletely described and its possible interactions with genes on Hsa21 are unknown. The increased incidence of OA in Ts, $Tbx5^{+/-}$ mice may be caused by an interaction between *Tbx5* and a trisomic gene(s) that is regulated by this transcription factor. *Tbx5* target genes have been identified via high throughput methods but few have been verified [82]. The few verified transcriptional targets of *Tbx5* include *Fgf10* [83, 84], *Gja5* [42], and *Anf* [42, 49]. *Tbx5* has also been implicated in the transcriptional regulation of *Gata4* [42, 85] and *Hey2* [42], but these targets have yet to be verified. We sought to add to the list of *Tbx5* transcriptional targets by identifying trisomic genes that may be regulated by *Tbx5.* Trisomic genes with T-box binding elements (TBEs), a known role in heart development, and an association with the Tbx5 protein were identified, and their transcript levels in trisomic and euploid $Tbx5^{+/-}$ mice were examined.

Methods

Identification of candidate genes

One hundred nine (109) genes that are trisomic in Ts65Dn mice were examined for localization of expression in the heart using the following databases: EMAGE gene expression database (http://www.emouseatlas.org/emage/home.php), VisiGene image browser (http://genome.ucsc.edu/cgi-bin/hgVisiGene?hgp_listSpec=), the Chromosome 21 gene expression atlas (http://ch21exp.tigem.it/), and the MGI gene expression

database (http://www.informatics.jax.org/expression.shtml). The 64 trisomic genes expressed in heart during development, according to the above databases, were compared to a list of genes bound by Tbx5 in a ChIP study [86]. A list of 43 trisomic candidate genes that were expressed in heart and bound by Tbx5 was then generated (Figure 7, Table 5). From the list of 43 genes, 6 were chosen as top candidates. The top 6 genes were the only genes out of the 43 which had TBEs in their promoter regions, were found to be bound by other heart specific transcription factors, had high evolutionary conservation in the TBE containing regions, and were previously implicated in heart development. The Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess) [87] was used to search for the TBEs (RGGTGTVR) [88].

Real-Time Analysis of Gene Expression

Total RNA was extracted from the hearts of E11.5 embryos using the RNeasy mini kit (Qiagen). Heart tissue from 3-6 embryos of each genotype was pooled together. cDNA synthesis was carried out with the First-Strand cDNA synthesis kit (Life Sciences) using 1 μg of total RNA as template. PCR was carried out on a 7500 Real-Time PCR System (Applied Biosystems) using POWER SybrGreen master mix (Applied Biosystems). 50 ng of cDNA was used in each PCR reaction. Each PCR run consisted of three technical replicates, and at least three biological replicates were performed. Primers used are as follows: Dyrk1a-F: 5`-AGGTGCGCCAGCAGTTTCCG-3`; Dyrk1a-R: 5`-ATGCAATGCGTTCTGCTGG-3`; Nfatc1-F: 5′-CGGCGCAAGTACAGTCTCAATGGCG-3′; Nfatc1-R: 5′-GGATGGTGTGGGTGAGTGGT-3′; Adamts1-F: 5’-CACGTGTGACACTCTCGGAA-3’; Adamts1-R: 5’-CGTGCGGCATGTTAAACACA-3’;

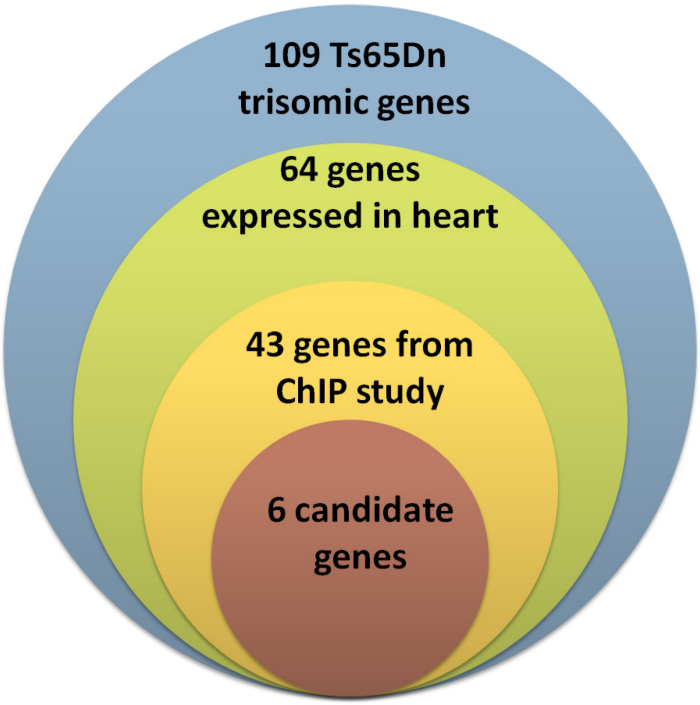


Figure 7. Identifying trisomic gene candidates for Tbx5 interaction. Gene expression database were searched to find expression domains of all Ts65Dn trisomic genes. Genes that were expressed in the heart during development were further investigated for interaction with Tbx5 using data from a ChIP study. Of the 43 genes associated with Tbx5 in the ChIP study 6 were chosen as candidate based on the appearance of a T-box binding element in the gene.

Table 5. Forty-three Ts65Dn trisomic genes expressed in heart during development and bound by Tbx5 in a ChIP experiment

Ts65Dn genes identified by ChIP with Tbx5[a]			
Adamts1	*Dopey2*	*Il10rb*	*Ripply3*
Adamts5	*Dscr3*	*Itsn1*	*Rwdd2b*
App	*Dyrk1a*	*Jam2*	*Sh3bgr*
Atp5j	*Erg*	*Kcne1*	*Setd4*
Atp5o	*Ets2*	*Morc3*	*Sod1*
Bace2	*Gabpa*	*Mrap*	*Son*
Bach1	*Gart*	*Mrpl39*	*Tmem50b*
Cbr1	*Hmgn1*	*Mrps6*	*Ttc3*
Cbr3	*Hunk*	*Prdm15*	*Usp16*
Cryzl1	*Ifnar1*	*Psmg1*	*Wrb*
Donson	*Ifnar2*	*Rcan1*	

[a] He, A., et al., *Co-occupancy by multiple cardiac transcription factors identifies transcriptional enhancers active in heart.* Proc Natl Acad Sci U S A, 2011. **108**(14): p. 5632-7

Rcan1-F: 5'-TTGTGTGGCAAACGATGATGT-3'; Rcan1-R: 5'-CCCAGGAACTCGGTCTTGT-3'; Gapdh-F: 5'-TGCACCACCAACTGCTTAG-3'; Gapdh-R: 5'-GATGCAGGGATGATGTTC-3'. The PCR was done under the following cycling conditions: 95˚C 15 min, (95 ˚C 15s, 60˚C 1 min) for 40 cycles, 95 ˚C 15 sec, 60°C 1 min, 95 ˚C 15 sec. Expression of candidate genes was normalized to *Gapdh.*

Creation of Tbx5 constitutively over-expressing cells

A *Tbx5* cDNA clone was obtained from the I.M.A.G.E. consortium and subcloned into the pcDNA3.1+ vector (Life Technologies), putting it under the control of the CMV promoter. NIH-3T3 cells were transfected with the pcDNA-Tbx5 construct using Lipofectamine 2000 (Life Technologies). Stably transfected cell lines were selected with 1 mg/ml Geneticin (Gibco). The cells were maintained in Dulbecco's Modified Eagle Medium with high glucose (Life Technologies), 10% fetal bovine serum, 1 mg/ml Geneticin, 1X glutamine and penicillin/streptomycin at 37°C and 5% CO_2.

Luciferase Assay

The promoter region of *Adamts1* that was bound by Tbx5 in a ChIP study [86] was amplified by PCR with the insertion of cut sites for *KpnI* and *XhoI*. PCR primers are as follows, F: 5'-GGCGCTTATGGTACCTGGTCACACTTTTTTTGG-3'; R: 5'-GGCGCTTATCTCGAGCACCTTCACAGAGGCTCA-3'. The amplified region was subcloned into the pGL3 basic vector (Promega). Tbx5 siRNA and scrambled siRNA were purchased from Life Technologies. The constructs and siRNA were transfected into NIH-3T3 cells constitutively over expressing *Tbx5* using Lipofectamine 2000 (Life Technolgies). Luciferase levels were measured in a 1450 Wallac Jet MicroBeta liquid

scintillation and luminescence counter (Perkin-Elmer) using the Dual Luciferase Assay Kit (Promega).

Statistical Analysis

The relative quantification of gene expression and the luciferase assays comparing different constructs were compared by Student's t-test. At least three biological replicates for the quantification of gene expression were compared, with each biological replicate consisting of three technical replicates. All tests were 2-tailed, and $P<0.05$ were considered significant.

Results

Candidate trisomic genes interaction with Tbx5

The simplest explanation for the increased incidence of specific defects in Ts, *Tbx5*$^{+/-}$ mice is an interaction between *Tbx5* and a trisomic gene(s) that is regulated by this transcription factor. We considered the 109 genes that are trisomic in Ts65Dn mice and generated a list of potential candidate genes based on expression patterns (Figure 7). Sixty-four of these genes are expressed in the heart during development. Of those 64 genes, 43 contained regions that associated with Tbx5 in a ChIP study [86]. However, examination of these 43 revealed a T-box consensus binding site in only six. Based on prior reports of possible effects on heart development, evolutionary conservation, the presence of T-box binding sites, and binding sites for other heart specific transcription factors, we selected three of the six, *Dyrk1a*, *Rcan1*, and *Adamts1,* for further investigation. The Tbx5 protein binds at multiple sites in the promoter regions of all

three [86]. Using TESS, predicted T-box binding sites were found in the first exons and/or promoter regions of those genes [87].

Trisomy and *Tbx5* haploinsufficiency affect expression of genes in the NFAT pathway

Quantitative PCR was used to determine transcript levels of trisomic genes that are regulated by *Tbx5,* in hearts of trisomic and euploid, $Tbx5^{+/-}$ embryos at E11.5. We also examined expression of the non-trisomic *Nfatc1* gene because its activity is regulated by two of our candidate trisomic genes (Figure 8) [89, 90], and its expression is altered when *Tbx5* is over expressed [82]. Levels of *Nfatc1* were increased in Eu, $Tbx5^{+/-}$ mice compared to wild type controls (p=0.003) (Figure 9). In contrast, expression of *Nfatc1* in Ts, $Tbx5^{+/-}$ mice was reduced (p=0.01). *Dyrk1a* was up regulated in both Ts65Dn (p=0.02), and $Tbx5^{+/-}$ mice (p=0.01) (Figure 9), reflecting separate influences of both trisomy and *Tbx5* dosage on expression of *Dyrk1a*. Surprisingly, expression levels of *Dyrk1a* in Ts, $Tbx5^{+/-}$ mice were half of the wild type level (p=0.002) (Figure 9). Only the increase in *Rcan1* levels in $Tbx5^{+/-}$ mice compared to euploid animals was statistically significant (p=0.01) (Figure 9).

The data suggests that Tbx5 represses genes in the NFAT pathway. Although trisomy alone does not affect the pathway, when the two factors are present together, transcription of NFAT pathway genes is repressed. The interaction of *Tbx5* and trisomy has an effect on gene transcription that is not seen in trisomy alone or *Tbx5* haploinsufficiency alone. *Tbx5* haploinsufficiency, either directly or indirectly, alters expression of a trisomic gene or genes which further impede normal development of the

Figure 8. Role of trisomic genes *Rcan1 (Dscr1)* and *Dyrk1a* in regulation of the translocation of Nfatc1 protein within the cell. Entry of calcium into the cell leads to the activation of calcineurin (Cn) and the de-phosphorylation of Nfatc family members. *Rcan1* binds Cn to inhibit its de-phosphorylation of Nfatc1 and prevents Nfatc nuclear entry. De-phosphorylation of Nfatc1 by Cn allows the protein to move into the nucleus. Once in the nucleus Nfatc1 induces transcription of several genes including *Nfatc1, Dyrk1a,* and *Rcan1*. Dyrk1a aids in phosphorylating Nfatc1, promoting its movement out of the nucleus. Others have proposed that an increase in *Rcan1* and *Dyrk1a*, which is what is expected to occur in trisomy 21, will cause a slower entry and a quicker exit of Nfatc family members from the nucleus [90]. Reprinted by permission from Macmillan Publishers Ltd: Arron, J.R. *et al,* NATURE 441(7093): 596-600 copyright (2006).

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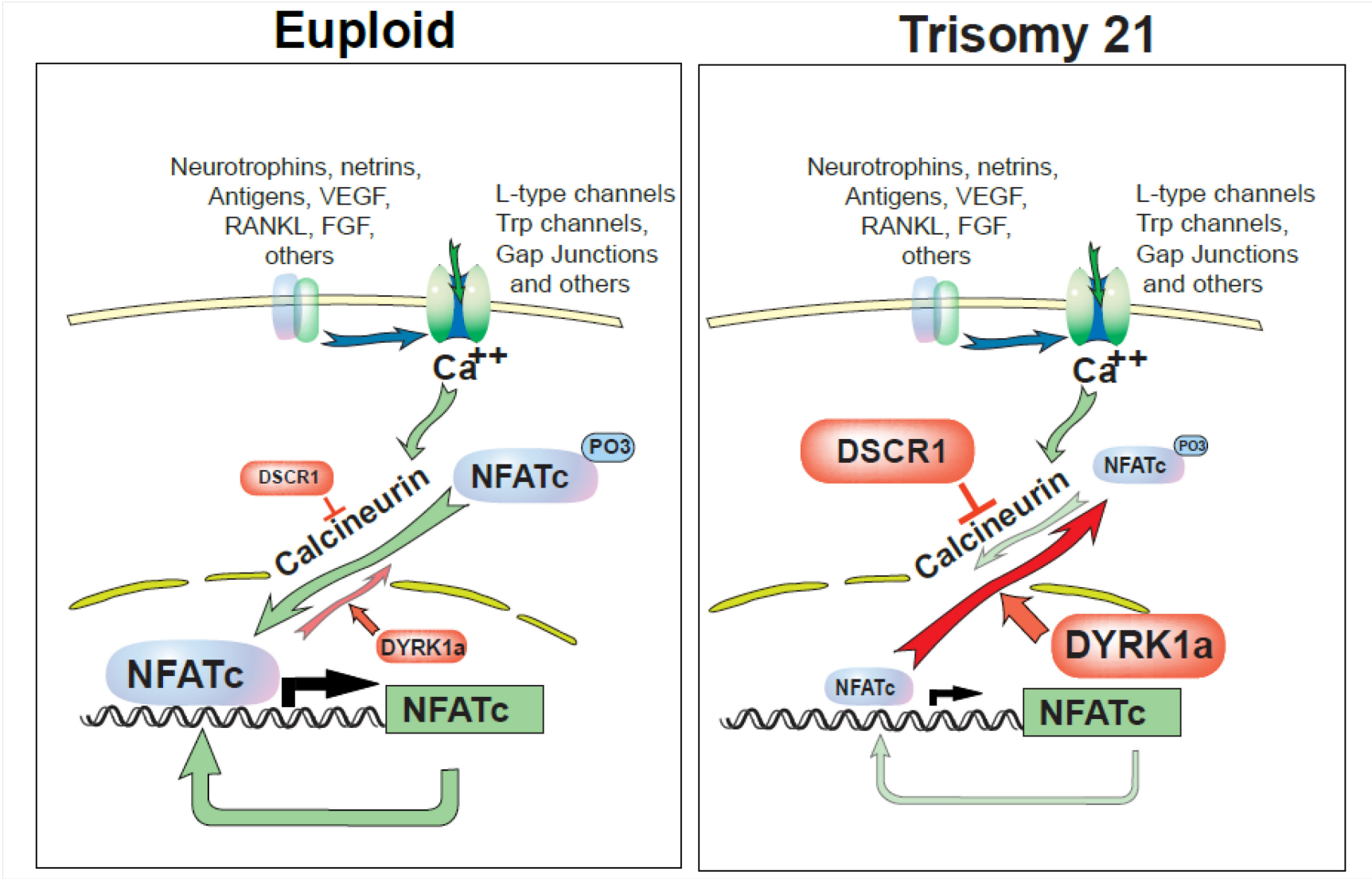
Euploid
Neurotrophins, netrins,
Antigens, VEGF,
RANKL, FGF,
others
L-type channels
Trp channels,
Gap Junctions
and others
Ca++
DSCR1
Calcineurin
NFATc
PO3
DYRK1a
NFATc
NFATc
Trisomy 21
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others
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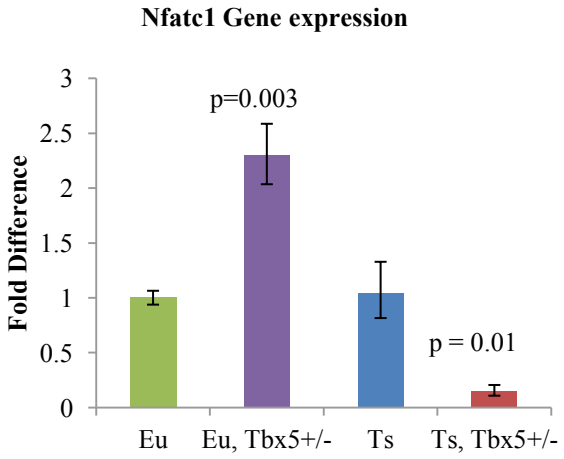
Gap Junctions
and others
Ca++
DSCR1
Calcineurin
NFATc
PO3
DYRK1a
NFATc
NFATc

Figure 9. Quantitative PCR of candidate genes in E11.5 hearts. Quantitative PCR of candidate gene expression levels in 3-6 pooled E11.5 embryonic hearts. Expression levels of Nfatc1 (A), Dyrk1a (B), Rcan1 (C), and Adamts1 (D). Levels of *Nfatc1* are significantly decreased in Ts, $Tbx5^{+/-}$ mice (0.2x, p= 0.01) and increased in euploid $Tbx5^{+/-}$ mice (2.3x, p= 0.003). Transcript levels of *Dyrk1a* are significantly increased in $Tbx5^{+/-}$ (4.6x, p= 0.01) and Ts65Dn mice (2.4x, p= 0.02). In the trisomic $Tbx5^{+/-}$ mice, *Dyrk1a* is decreased by half compared to WT mice (p= 0.002). For *Rcan1* the only significant difference is between the WT and euploid $Tbx5^{+/-}$ mice. *Rcan1* expression is up regulated 1.7x in the $Tbx5^{+/-}$ mice (p=0.01). For the *Adamts1* assay levels are significantly increased in euploid $Tbx5^{+/-}$ mice (1.95x, p= 0.02). Error bars are standard deviation.


Nfatc1 Gene expression
Fold Difference
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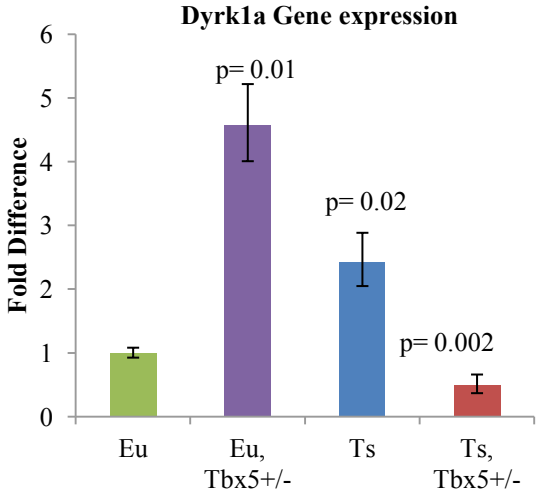
2.5
2
1.5
1
0.5
0
p=0.003
p = 0.01
Eu
Eu, Tbx5+/-
Ts
Ts, Tbx5+/-


Dyrk1a Gene expression
Fold Difference
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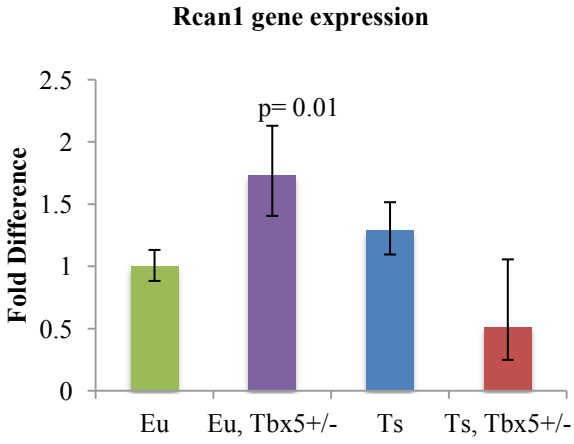
5
4
3
2
1
0
p= 0.01
p= 0.02
p= 0.002
Eu
Eu, Tbx5+/-
Ts
Ts, Tbx5+/-


Rcan1 gene expression
Fold Difference
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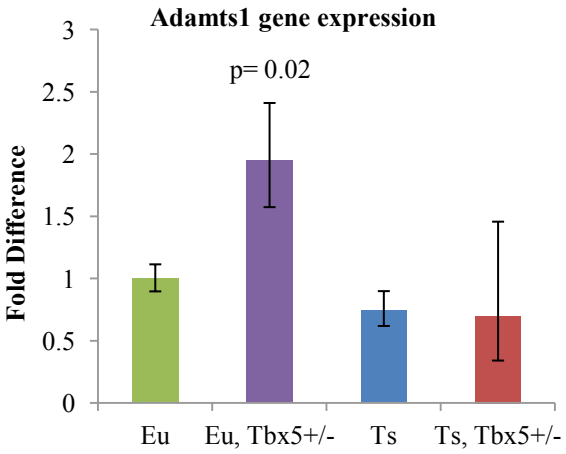
2
1.5
1
0.5
0
p= 0.01
Eu
Eu, Tbx5+/-
Ts
Ts, Tbx5+/-


Adamts1 gene expression
Fold Difference
3
2.5
2
1.5
1
0.5
0
p= 0.02
Eu
Eu, Tbx5+/-
Ts
Ts, Tbx5+/-

heart. Our data suggests that the NFAT pathway is affected during development of Ts, *Tbx5*$^{+/-}$ mice.

Tbx5 dosage and binding alters transcription of *Adamts1*

Adamts1 transcript levels were significantly increased in Eu, *Tbx5*$^{+/-}$ mice (p=0.02) (Figure 9), suggesting that Tbx5 acts as a repressor of *Adamts1*. Changes in gene expression for the other genotypes did not reach statistical significance. We tested a putative Tbx5 binding site identified in the *Adamts1* locus [86] using a luciferase reporter assay. A 238 bp region located 121 bp upstream of the *Adamts1* transcription start site was amplified by PCR and cloned into the pGL3 luciferase vector (Figure 10). This construct was transiently transfected into cells stably over expressing *Tbx5* and luciferase levels were measured. We found a significant increase in luciferase levels when comparing the pGL3 transfected cells with those transfected with the Adamts1 -121 construct (p=0.00014) (Figure 11). Introduction of siRNA directed at Tbx5 reduced this effect (p=0.006). Thus while the overall effect of Tbx5 is repressive, binding of Tbx5 at the Adamts1 -121 region alone positively regulates transcription.

Discussion

We observed effects of both Tbx5 and trisomy on the NFAT signaling pathway, which is important for proper development of the cardiac septa, valves, and OFT [91-93]. *Dyrk1a* and *Rcan1* are trisomic inhibitors of NFAT signaling [89, 90, 94] (Figure 8), and their over expression down regulated Nfatc1 protein expression in E13.5 mouse embryos [90]. Our q-PCR data show trisomy or reduction in Tbx5 both resulted in increased transcript levels of *Nfatc1, Dyrk1a,* and *Rcan1*. This relationship was more

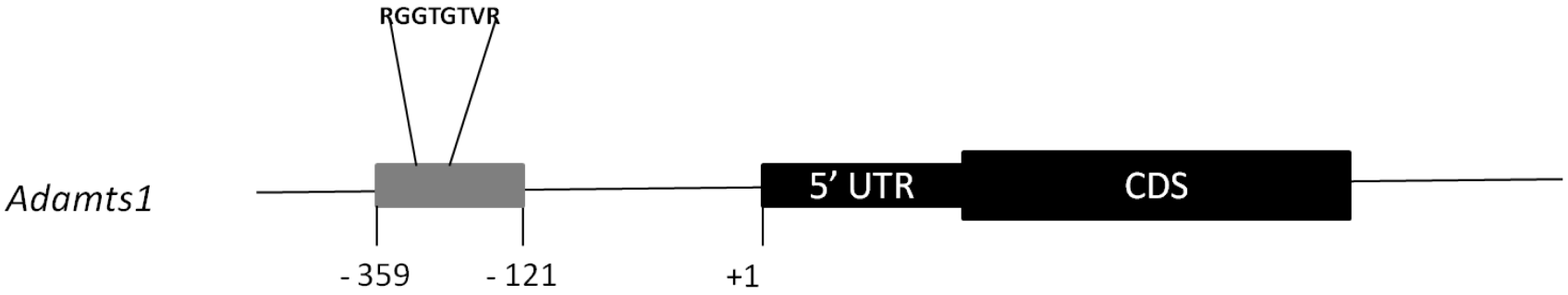
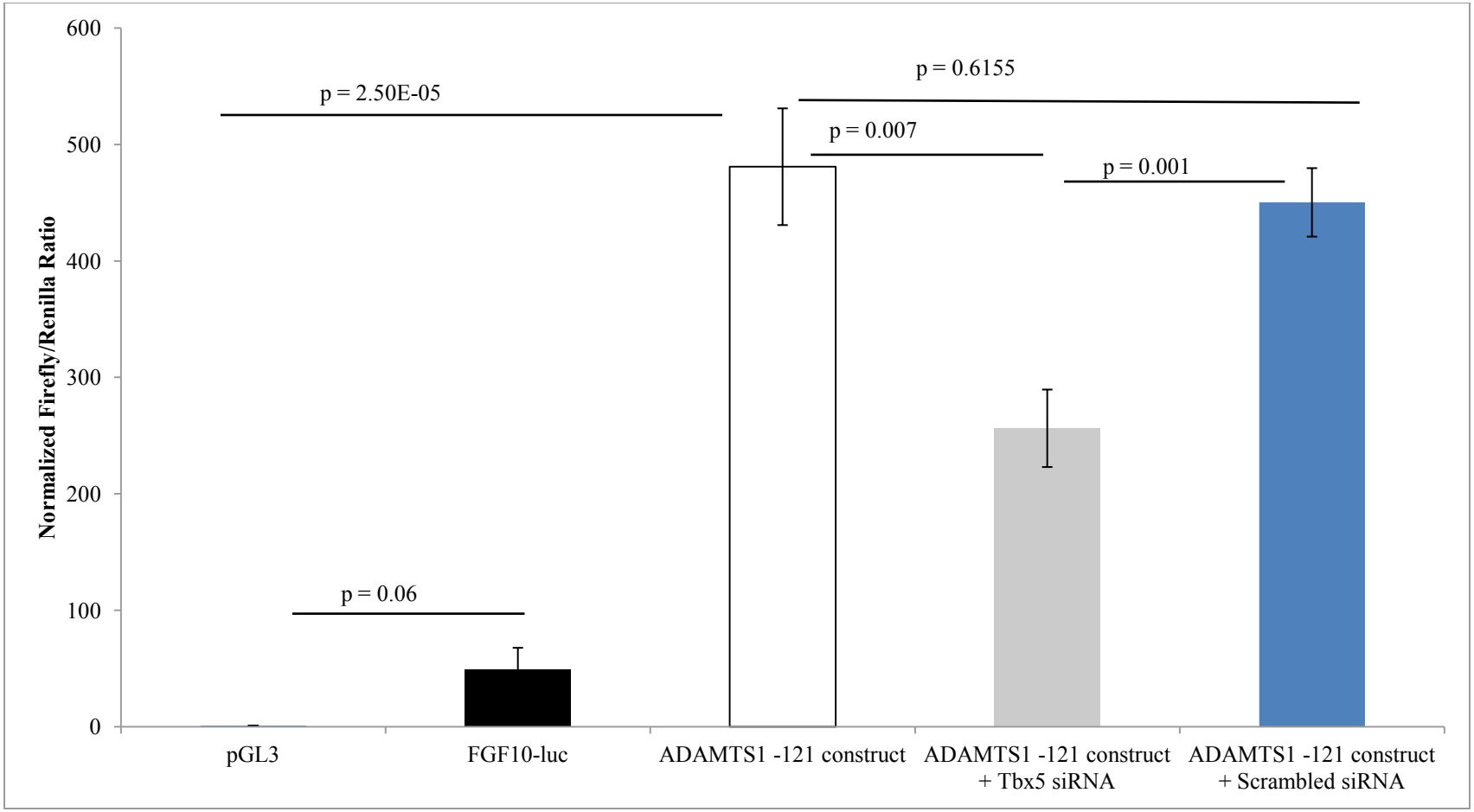


Figure 10. Region of *Adamts1* gene inserted into pGL3 vector. The region highlighted in gray contains the consensus T-box binding site (RGGTGTVR) and was amplified by PCR and cloned into a pGL3 luciferase vector (Promega). This region was associated with Tbx5 in a ChIP study [86].

Figure 11. Effects of Tbx5 over expression on transcription of an *Adamts1* luciferase construct. All constructs showed significantly higher luciferase expression when compared to the pGL3 luciferase construct backbone. Cells transfected with the Adamts1 -121 construct and a Tbx5 siRNA showed significantly lower luciferase levels when compared to those transfected with the -121 construct alone ($p=0.007$), compared to ADAMTS1 -121). Graph represents one experiment representative of all replicates. The region inserted into the basic pGL3 vector is 121 bp upstream of the *Adamts1* gene and associated with Tbx5 in a ChIP study. Tbx5 over expressing cells were harvested 48 hours after transfection. The FGF10-luciferase construct was used as a positive control. pGL3 construct was normalized to 1. Error bars are standard deviation.


Normalized Firefly/Renilla Ratio
600
500
400
300
200
100
0
p = 2.50E-05
p = 0.6155
p = 0.007
p = 0.001
p = 0.06
pGL3
FGF10-luc
ADAMTS1 -121 construct
ADAMTS1 -121 construct + Tbx5 siRNA
ADAMTS1 -121 construct + Scrambled siRNA

complex in the presence of trisomy; reduced expression was seen when the *Tbx5* null allele occurred in trisomic mice. There are two likely reasons why our results differ from the previous study. In the previous studies [90] only *Dyrk1a* and *Rcan1* were up regulated, causing down regulation of the Nfatc1 protein, but many more genes were up regulated in our trisomic mice and our study examined transcript levels not protein. It is clear that alterations in NFAT signaling correlate with the combination of trisomy and *Tbx5* haploinsufficency. This is evidence that *Tbx5* dosage can have a significant effect on the NFAT pathway on the background of trisomy.

Questions that arise when considering these data are, "What specific effect is the combination of *Tbx5* haploinsufficiency and trisomy having?," and "What mediates that effect?" Trisomic mice have three copies of *Rcan1* so transcriptional activation of this gene would result in negative regulation (i.e., repression) of NFAT signaling. Since NFAT signaling regulates EMT in the endocardial cushions it is likely that modulation of the NFAT pathway by either trisomy or *Tbx5* dosage could result in an error during development of the cardiac cushions. Indeed, loss of *tbx5* in zebrafish leads to an increase in valve tissue, while knockouts of *Nfatc1* in mice show stunted or absent valves, as well as VSDs, suggesting that the increase in *Nfatc1* due to knockdown of Tbx5 could play a role in the defects seen in the mice described here [92, 93, 95]. Interestingly, cardiac defects in zebrafish with mutations in *tbx5* are rescued by addition of cytosolic CAMK-II [96], which inhibits the NFAT pathway by interacting with calcineurin. In rat ventricular myocytes, expression of a constitutively active CAMK-II protein inhibited nuclear translocation of Nfatc3 [97]. Reduction in dosage of *Tbx5* could lower CAMK-II levels, causing a reduction in the negative regulation of calcineurin by CAMK-II. *Tbx5*

may regulate expression of CAMK-II which in turn negatively regulates NFAT signaling. This model would explain the increase in expression of *Nfatc1* in mice heterozygous for the *Tbx5* null allele. An investigation into CAMK-II levels and Nfatc1 protein localization in these mice would be essential to investigate this hypothesis further.

Our data also show that *Tbx5* dosage affects the expression of the *Adamts1* gene. *Adamts1* is important in the development of the extracellular matrix in the endocardial cushions, and is a regulator of the VEGF pathway. Reports show that *Adamts1* blocks proliferation initiated by the VEGF pathway and also blocks Vegf from binding to its receptor [98]. An excess of variants in VEGF pathway genes is associated with DS-related AVSD [25]. Binding of Tbx5 to the *Adamts1* promoter induces expression in luciferase assays while haploinsufficiency of *Tbx5* alters expression of *Adamts1* in E11.5 hearts, showing that Tbx5 has a role in the regulation of *Adamts1* transcription. When Tbx5 siRNA was added expression was down regulated; although, the addition of Tbx5 siRNA did not return the luciferase levels to that of the control. These results indicate that there may be another factor that co-regulates *Adamts1* expression along with Tbx5. Expression of *Adamts1* is repressed by Brg1, a protein that complexes with Tbx5 [99], and binds the promoter region of the *Adamts1* gene [100]. Studies of $Brg1^{+/-};Tbx5^{+/-}$ mice found evidence that the two proteins interact *in vivo*, and that reduced dosage of *Tbx5* results in reduced occupancy of Brg1 at known *Tbx5* target genes [22]. The authors suggest a model based on allelic balance between Tbx5 and Brg1 that would explain the results seen here. When the two alleles are unbalanced each has the opportunity to bind with other factors, including hypothetical repressors and activators. In accordance with this model reduced dosage of *Tbx5* would allow Brg1 to interact with other factors,

potentially altering gene transcription, and increased dosage of *Tbx5* would allow Tbx5 to interact with other factors, again altering gene transcription.

Our quantitative PCR results suggest that Tbx5 may act as a repressor of *Adamts1* expression *in vivo* (Figure 8). The contradiction in the results of our quantitative PCR and luciferase assays can be explained by the nature of the experiments; the PCR reflects *in vivo* levels while the luciferase assays were done in cell culture. Only a portion of the region upstream of *Adamts1* was included in the luciferase construct but other regions are relevant during transcription. The presence of these other regions may alter transcription of *Adamts1*. Overall, our data show that *Tbx5* is involved in the transcriptional regulation of the *Adamts1* gene, a regulator of the VEGF pathway. We have shown that a change in Tbx5 dosage can affect transcription of several trisomic genes, which are components of the NFAT and VEGF signaling pathways. The links that we demonstrated between Tbx5 and the NFAT pathway as well as the *Adamts1* gene suggests new targets for Tbx5 and molecular mechanisms underlying CHD.

Chapter 4: A universal response deficit to Sonic hedgehog (Shh) in DS and its possible role in CHD

(Adapted from Currier, D.G., Polk, R.C., and Reeves, R.H. A sonic hedgehog (Shh) response deficit in trisomic cells may be a common denominator for multiple features of Down syndrome. Progress in Brain Research. 2012;197:223-36.)

The first direct demonstration of Shh response perturbation due to trisomy came from analysis of cerebellar development in the Ts65Dn mouse [101]. Ts65Dn mice, like people with trisomy 21, have a smaller cerebellum and show specific deficits of Purkinje cells and of the granule cell neurons that make up the internal granule layer (IGL) of the cerebellum. Further, the reduced density of granule cells in the IGL of Ts65Dn mice was shown to occur in people with DS, as well [101]. Purkinje cells produce Shh which stimulates GCPs to divide and migrate inward to form the IGL [102-104]. The frequency of mitosis is significantly reduced in Ts65Dn, and this reduced mitotic rate is a major contributor to the deficit in granule cell generation in trisomic mice [105] and in DS [106]. Similarly, deleting a floxed Shh gene in late gestation by driving Cre expression with either the *Pax2* or *L7* promoters results in reduced cerebellar volume, hypocellularity and disorganization of GCPs in the external germinal layer (EGL) of the cerebellum [107]. When GCPs were isolated from trisomic and euploid cerebella and cultured in the presence of increasing amounts of Shh, trisomic GCP responded less to the mitogenic effects of Shh [105].

These results raise the question, “Is the attenuated response to Shh in trisomic mice restricted to GCP, or do all Shh-responsive cells in a trisomic individual show a reduced reaction to Shh stimulation?” Observations of parallel effects of Shh disruption

and of trisomy suggest that this mechanism may contribute to multiple DS phenotypes. Based on the demonstration that trisomy results in a reduced response to the mitogenic effects of Shh in cerebellum, the possible effects of attenuated Shh response in heart were considered here. We consider possible effects on heart development if the Shh responding cells "saw" less Shh signal as is the case for GCPs.

The Shh ligand is produced in cells distinct and often separated from those receiving the signal. Extracellular Shh-Np is sensed by the receiving cell via interactions with the 12-pass transmembrane protein, Patched (Ptch) [108, 109]. In the pathway-off state (Fig. 12a), Ptch catalyzes the production of an unidentified repressor of Smoothened (Smo), a 7-pass transmembrane protein with possible G-protein coupled receptor activity [110, 111]. When Smo is repressed (pathway-off), the transcription factors Gli2 and Gli3 are targeted to the proteasome for processing to produce their transcriptional repressor forms ($Gli2^R$, $Gli3^R$) [112, 113]. Another pathway element, Suppressor of Fused (SuFu), is found in both the cytoplasm and the nucleus and interacts with Gli1 and Gli2 to further suppress pathway activity [114, 115]. SuFu/Gli complexes are exported from the nucleus and tethered in a SuFu dependent manner, in the cytoplasm. Further, SuFu inhibits Gli mediated transcriptional activation by binding and inhibiting DNA-bound Gli1 or Gli2. The pathway is activated when Shh binds to Ptch, inhibiting the catalytic activity of the latter, thereby reversing the repression on Smo (Fig. 12b). This results in degradation of SuFu and Gli phosphorylation to produce activator Gli proteins that move to the nucleus and promote transcription [110, 116-118].

Jenkins [119] broadly defines several mechanisms for pathway activation outside of the canonical de-repression of Gli transcription factors following Shh binding to Ptch.

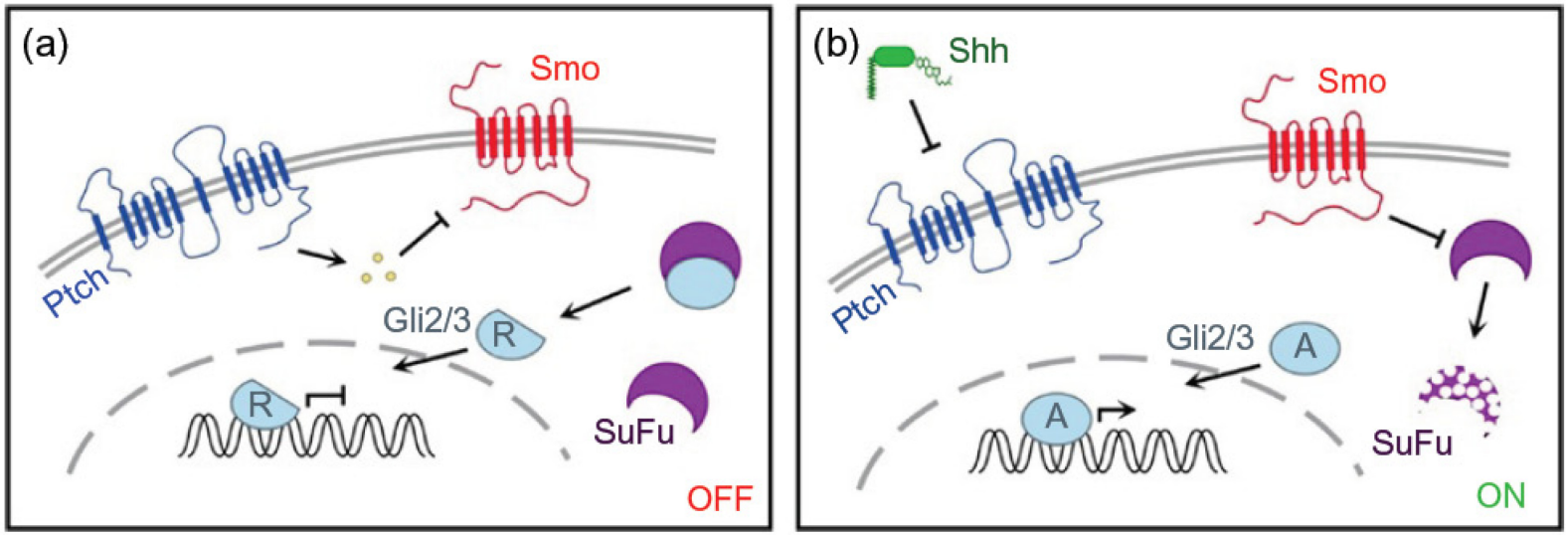


Figure 12. The Sonic hedgehog pathway. (a) In the pathway-off state, Patched (Ptch) catalytically inhibits Smoothened (Smo) activity through an unidentified intermediate. Suppressor of Fused (SuFu) mediates cleavage of Gli2 and Gli3 into their repressor forms, lacking transactivation domains. The Gli2/3 repressors translocate to the nucleus where they repress transcription by binding target gene promoter sequences. (b) Shh binding to Ptch inhibits its catalytic repression of Smo, resulting in activation of Smo and degradation of SuFu. In the absence of SuFu, Gli2/3 are phosphorylated to become the activator forms. Gli2/3 activators translocate to the nucleus where they promote transcription by recruiting other transcriptional activators to target gene promoters.

For example, Ptch can interact directly with CyclinB1 to affect cell cycle progression [120] and can initiate apoptosis in neuroepithelial cells until it is blocked by Shh binding [121]. Although Ptch is the primary receptor for Shh, several other membrane bound proteins compete for Shh and are capable of enhancing or inhibiting pathway activity. Cell Adhesion Molecule-Related/Down-Regulated by Oncogenes (*CDO*), Brother of CDO (*BOC*), Growth Arrest Specific 1 (*Gas1*) [122], and Hedgehog Interacting Protein (*Hhip*) [123] all interact with Shh. Of these, expression of CDO, BOC, and Gas1 increases Shh pathway activity, while Hhip negatively regulates the pathway [124]. The Gli transcription factors can also be regulated outside of the canonical Shh pathway. Borycki et al demonstrated that Wnt1 and Wnt4 can induce *Gli2* expression and repress *Gli3* expression in a quail segmental plate mesoderm explant culture system [125]. Others have suggested that Gli1 protein may be regulated independently of Shh through the MAPK pathway [126].

Although much of the work regarding the Shh pathway has examined brain development, the pathway also plays a role in heart development. Shh is secreted from cells in both the pulmonary endoderm, where it is required for proper atrial septation [127], and in the pharyngeal endoderm, where it is necessary for proper OFT septation [128]. Shh signaling marks cells within the SHF as progenitors of the atrial septum and OFT. Labeling of hedgehog-responsive cells early in heart development demonstrates that those cells migrate from the SHF and contribute to the primary atrial septum, dorsal mesenchymal protrusion (DMP), endocardial cushions and pulmonary trunk [127]. The atrial septum, DMP, and endocardial cushions all combine to form the mesenchymal

complex of the atrioventricular septum [129]. The appearance of this complex is necessary to complete AV septation and to anchor AV valves.

Neural crest cells (NCCs) of the fourth and sixth pharyngeal arches contribute to heart development by migrating into the OFT of the heart, contributing to septation and alignment. *Smo* is necessary for Shh pathway activation, and the loss of this gene in NCC resulted in errors in septation and alignment of the aorta and pulmonary trunk, as well as defects in pharyngeal arch arteries [130]. NCCs contribute substantially to the first pharyngeal arch (PA1), and trisomic embryos have been shown to have a smaller PA1, which contain fewer neural crest-derived cells and these cells had a lower mitotic index than did their euploid counterparts [131]. Several steps in NCC delamination, migration and proliferation require Shh signaling. Trisomic cells isolated from PA1 showed lower proliferation than did euploid. However, addition of Shh to the cultures increased cell division, bringing the rate in trisomic cells to that seen in euploid cultures [132]. Trisomic neural crest cells in PA1 exhibit defects in proliferation, which can be rescued by Shh. If these defects extend to NCC of PA 4 and 6 then it is likely the reduced response to Shh is contributing to CHD in trisomy. A Shh response deficit could thus contribute to heart defects through direct effects in SHF, or because of an impaired response of trisomic neural crest.

In support of this idea, several mouse models with impaired Shh signaling also display errors in heart development (Table 6). A knockout of *Shh* (*Shh*$^{-/-}$), in which exon 2 and its flanking introns are removed, displays AVSD and other structural defects [133, 134]. Similarly, when Shh signaling is blocked by cyclopamine at HH stage 14 chick embryos, persistent truncus arteriosus (PTA), VSD and pulmonary atresia secondary to

Table 6. Congenital heart defects in Sonic hedgehog pathway mutants

Perturbation	Phenotypes	Age	Notes	Refs.
Shh$^{-/-}$	Pharyngeal arch artery defects, ASD, VSD, Tetralogy of Fallot-like, Persistent truncus arteriosus (PTA)	E10.5 – E15.5		[133, 134]
Nkx2.5$^{Cre/+}$; *Shh*$^{flox/-}$	Pharyngeal arch artery defects, PTA, AVSD	E10.5	*Shh* ablated in *Nkx2.5* expressing cells	[128]
Mef2c-AHF-Cre; *Smo*$^{flox/-}$	PTA, ASD, VSD, AVSD, rounded and short AV valves	E14.5 – E18.5	*Smo* ablated in anterior heart field	[130]
Wnt1-Cre; Smo$^{flox/-}$	PTA	E10.5	*Smo* ablated in neural crest	[128]
Smo$^{Gli1\text{-}CreERT2}$	ASD and AVSD	E13.5	Floxed *Smo* allele under the control of inducible *Gli1:Cre*	[127]
Shh$^{Nkx2.1\text{-}Cre}$	ASD	E13.5	*Shh* ablated in *Nkx2.1* expressing cells	[127]

reduced proliferation in the SHF result [135]. Analogous outcomes occur when other components of the pathway are altered. Conditional knockouts of *Smo* and *Shh* result in AVSD and PTA in mouse embryos. Deletion of a floxed *Shh* allele in all cells expressing either *Nkx2.5* or *Gli1* results in AVSD [127, 128]. Thus Shh signaling mutants present AVSDs, VSDs, and ASDs, structural defects that are common in DS [136].

Septal defects were attributed primarily to errors in the endocardial cushions for many years, but evidence has emerged that points to a critical role for DMP as a contributing factor, especially to AVSD and secundum ASD [127, 130]. In this light, it is relevant that Shh signaling is not required for endocardial cushion contributions to septation, but is necessary for proper contributions to DMP from the SHF. When Shh signaling is disrupted in DMP progenitors or the SHF, the DMP is hypoplastic or does not form and an AVSD results [127, 130]. Hypoplastic DMP has also been described in human fetuses with DS and in mice trisomic for all of Mmu16 [129, 137, 138]. Hence, there is an important role for Shh signaling in formation of the DMP, and for DMP involvement in AVSDs; DS is the biggest major risk factor for AVSD [136]. Overall, there are substantial similarities between heart phenotypes caused by trisomy and those seen in Shh signaling mutants. These results do not prove causation but they are consistent with the effects expected from reduced response to Shh signaling in the developing heart.

None of the genes encoding canonical Shh signaling pathway components is encoded on Hsa21. However, up-regulation of *Ptch* (resulting in down-regulation of the SHH pathway) has been reported in Ts65Dn mice for a specific, small group of stem cells in the sub-ventricular zone (SVZ), the origin of granule cells in the dentate gyrus [139].

In cultured neurospheres developed from the SVZ region, a C-terminal fragment of the APP protein, AICP, can contribute to the up-regulation of *Ptch* transcription [139]. Since the *APP* gene is found on Hsa21 and thus is chronically upregulated in DS (and also in Ts65Dn mice), this provides a possible explanation for the attenuated mitogenic response to Shh by trisomic cells.

Molecular pathway analysis has implicated several additional Hsa21 genes whose expression may impinge on Shh signaling directly or indirectly, especially on the regulation of *Gli1, 2* and/or *3* (reviewed in [140]). To date, however, there is no direct demonstration of a dosage sensitive trisomic gene disrupting Shh signaling in the developing heart. Trisomic mouse models of DS provide a sensitized genetic background for dissection of these mechanisms. Trisomy for Hsa21 results in increased dosage for more than 300 genes and numerous studies of gene expression in DS and in animal models suggest that most of these will be up-regulated by ~50% whenever and wherever they are normally expressed. The availability of segmental trisomies in animal models that recreate the dosage imbalances seen in DS and the demonstration that this produces features analogous to those in DS [141] has led to a productive phenotype-based approach to the development of therapies [141-143].

The phenotype-based approach suggests the possibility that multiple effects of trisomy in different tissues may result from perturbations in the same developmental pathways and regulatory processes, as posited here for Shh. A deficit in response to the mitogenic effects of Shh has been demonstrated in trisomic cerebellar GCP. Trisomic NCC-derived cells in PA1 also appear to respond less to Shh than do their euploid

counterparts. A similar response deficit in other trisomic cell types could affect development of the heart, and perhaps other tissues affected in DS.

Chapter 5: Conclusions

Trisomy 21 is a destabilizing condition. Many features of the syndrome are present in all individuals with trisomy 21, such as cognitive impairment and craniofacial dysmorphology; but it is not the cause of many other features, such as CHD. The absence of 70+ features in many individuals with DS is indication that DS is a major destabilizing factor in development but not the sole cause of many of the associated developmental errors. Evidence of the damaging effect of trisomy on heart development has been provided here.

Combining trisomy with haploinsufficiency for the *Tbx5* gene caused a dramatic increase in the appearance of a rare defect, OA. *Tbx5*$^{+/-}$ mice have an 18% incidence of OA, while Ts65Dn mice show a 17% frequency of defects affecting the great vessels [36]. Combining trisomy with a reduced dosage of *Tbx5* exacerbated the OA phenotype, resulting in 58% of Ts, *Tbx5*$^{+/-}$ mice having an OA. The combination of trisomy and reduced *Tbx5* dosage caused a dramatic increase in CHD, beyond what was seen when either factor was present alone. As mentioned in chapter 1, people with DS and mutations in *TBX5* show similarities in CHD with Ts, *Tbx5*$^{+/-}$ mice [66]. Both present with OA and many other defects. Similar insults in mice and humans show the usefulness of the Ts65Dn mouse model for the study of the genetics behind heart development. Analogous defects in mice and humans suggest that the molecular pathways involved in heart development are also comparable between these species.

Due to the large number of genes at dosage imbalance in trisomy, it is not surprising that multiple pathways are affected. We can begin to investigate the genetics underlying these defects in DS by studying the pathways involved in these mice. Multiple

genetic pathways that are critical to heart development are affected when trisomy and *Tbx5* haploinsuffiency are combined. The basis for the effect is complex. A change in Tbx5 dosage can affect transcription of *Adamts1, Dyrk1a,* and *Rcan1,* genes that are components of the NFAT and VEGF signaling pathways. Both pathways can be regulated by trisomic genes and are essential for proper heart development. The parallels between DS and Ts6Dn suggest that these pathways may also play a role in the development of CHD in people with DS. In fact, other studies have already shown links between DS-associated AVSD and components of the VEGF pathway [25] (Redig, J.K., G.T. Fouad, D. Babcock, B. Reshey, E. Feingold, R.H. Reeves, C.L. Maslen, Allelic Interaction between *CRELD1* and *VEGFA* in the pathogenesis of cardiac atrioventricular septal defects, AIMS Genetics, in press). Our findings document previously unknown connections between *Tbx5* and trisomic genes. We provide evidence of the usefulness of examining the impact of heart development mutations on a trisomic background to further understand the possible role of pathways in heart development.

Not only has this study provided evidence of the usefulness of the trisomic condition in studying the genetics of heart development it has also provided evidence of novel roles for both trisomy and *Tbx5* in heart development. The increased incidence of OA in Ts, $Tbx5^{+/-}$ mice suggest a role for both genetic insults in development of the OFT. Prior to this study defects in aortic alignment had not been seen in *Tbx5* mutants. OA has been identified in individuals with DS but only as a component of Tetralogy of Fallot. *Pitx2* expression is reduced on the Ts, $Tbx5^{+/-}$ background, and this could contribute to defects in the left-right asymmetry of the heart in these mice. Neither atrial isomerism nor left-right patterning defects have been associated with Ts65Dn or $Tbx5^{+/-}$ mice

previously. Putting *Tbx5*$^{+/-}$ on a trisomic background revealed an effect on left-right patterning. Destabilization induced by trisomy most likely results in a subclinical defect that exacerbates defects caused by reduced dosage of *Tbx5*.

While the mechanism for the increase in OA in Ts, *Tbx5*$^{+/-}$ mice is unknown, further study of the Ts65Dn mice could lead to the discovery of that mechanism. Evidence has been found linking *Tbx5* dosage to the Shh pathway. *Tbx5* is expressed in hedgehog responsive atrial septum progenitors, and ASDs in mice haploinsufficient for *Tbx5* were rescued by constitutive Shh signaling in the SHF [57]. As mentioned in Chapter 4, a universal response deficit to Shh in trisomic cells could be part of the mechanism behind the defects described here. The combinatory effect of reduced dosage of *Tbx5* and a reduced response to Shh signaling may lead to a further reduction in Shh signaling than either trisomy or *Tbx5* haploinsufficiency alone. Although the resulting defects described here and in the aforementioned study are not the same, the mechanism posited can still explain both defects. In the study by Xie *et al.* [57] the reduction of *Tbx5* dosage and the subsequent activation of the Shh pathway were specific to the SHF. The genetic insults in the Ts, *Tbx5*$^{+/-}$ mice were universal, affecting all cell types. An effect on Shh signaling in all cell types, rather than just the SHF, could explain the difference in defects in the two studies.

The OFT is the precursor to the aorta and pulmonary artery during development. It comprises distinct cell lineages: the primary heart field, the secondary heart field and the cardiac neural crest. Proper development of the OFT requires the cooperation of all these different regions. The cardiac neural crest cells migrate from pharyngeal arches 4 and 6 to populate the cardiac cushions of the OFT, aiding in alignment and septation.

Previous work in our lab has shown that Ts65Dn mice have a reduced number of cranial neural crest cells in pharyngeal arches 1 and 2, as well as deficits in delamination, mitosis, and migration due to a decreased response to Shh [131]. Similar deficits in the cardiac neural crest cells might contribute to the appearance of OFT defects in these mice. A reduced dosage of *Tbx5* in these hedgehog responsive cells may exacerbate the phenotypes described. *Tbx5* expression is an early marker of myocyte lineage. When cells from the secondary heart field begin to colonize the OFT, *Tbx5* is up-regulated, beginning the process of cardiomyocyte differentiation. Defects in the development of cardiomyocytes in the OFT are believed to lead to alignment defects [144]. Insufficiency of Tbx5 hinders development of cardiomyocytes, thus affecting the formation of the OFT.

The combination of *Tbx5* haploinsufficiency and a reduced response to Shh in trisomy may begin earlier in development than at the colonization of the OFT. In studies of the chick, *Pitx2* was induced by Shh very early in development. When an anti-Shh antibody was added to the left side of HH stage 5 chick embryos *Pitx2* expression was blocked in the lateral plate mesoderm, leading to laterality defects later in development [145]. Shh signaling is needed for proper expression of *Pitx2*. While no direct link has been found between *Tbx5* and *Pitx2* in the heart, connections between the two genes have been shown in another organ. *Pitx2* represses *Tbx5* in the abdominal wall of E10.5 mouse embryos [62]. If both reduced *Tbx5* dosage and a universal response deficit to Shh affect *Pitx2* expression then the left-right patterning defects in these mice could be explained.

The results reported here also remind us of the complexity of heart development, and of the coordination that must occur during this process. For example, ventricular septation requires not only the proper formation of the ventricular septum but also the endocardial cushions, the DMP, and other structures in the heart. A defect in any one of them could lead to improper septation. All of the different processes in the heart (i.e. chamber formation, septation, etc.) must coordinate with each other for proper heart formation. *Tbx5* is known to affect ventricular and atrial septation and *Pitx2* affects atrial development, yet the Ts, $Tbx5^{+/-}$ mice have an increase in defects of aortic alignment. These results show that atrial and ventricular development have an influence on aortic alignment. During embryological development all of the different processes that take place in the heart are working in conjunction and rely on each other for proper development.

DS is a leading risk factor for CHD, and clearly a destabilizing factor during development. Gene dosage imbalance as a result of trisomy has effects on Shh signaling, which interacts with other modifiers as well. The Ts65Dn mouse model shows phenotypes similar to those seen in DS, and provides an excellent model for studying genetic modifiers of CHD, both trisomic and disomic. The trisomic population is extremely useful for gaining a deeper understanding of heart development and CHD. Trisomy by itself weakens genomic stability, making it easier to unmask other modifiers of CHD.

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B.S. in Genetic Engineering, 2008, Chemistry minor, *magna cum laude* with honors, *Beta Beta Beta*.

RESEARCH EXPERIENCE:

Doctoral Research: Department of Physiology and the Institute of Genetic Medicine, Johns Hopkins University, 2008 – 2014.

Conducted research in the lab of Dr. Roger Reeves studying genetic modifiers of congenital heart defects. This research will help in understanding and treating congenital heart defects, the most common congenital anomaly in live births.

Undergraduate Research: Department of Biological Sciences, Cedar Crest College, 2005, 2007 - 2008. Conducted independent research in the lab of Dr. Audrey Ettinger studying gonadotropin-releasing hormone (GnRH)

and its relation to reproductive behavior in *Cichlasoma octofasciatum,* (Jack Dempsey) fish.

Collaborated on research in the lab of Dr. Audrey Ettinger studying the Monoamine oxidase (MAOA) gene and its relation to aggressive behavior in *Cichlasoma octofasciatum,* (Jack Dempsey) fish.

Both of these research projects helped in gaining a better understanding of the brain and its relation to behavior, which will aid in better understandings that link in humans.

Summer Undergraduate Research: The Jackson Laboratory**,** Bar Harbor, ME, 2007. Conducted research in the lab of Dr. Beverly Paigen studying genes related to HDL cholesterol. This research helped in understanding the underlying mechanisms that can lead some to have high cholesterol throughout their lives, while others do not exhibit this trait.

LEADERSHIP AND COMMUNITY SERVICE:

Participated in a panel discussion of career pathways for the Office of Youth Programs of the Washington, D.C. government. May 24, 2013.

Volunteer at Strawberry Knoll Elementary School, Gaithersburg, MD, 2004-2014. Assist 1st ,2nd, and 4th grade teachers teaching units on the life cycle and rocks, sand and soil every year. I come into the classrooms and do science activities with the students and speak to them about my research and answer any questions they may have.

Volunteer with The Second Mile organization for underprivileged youth ages 4-12, Cedar Crest College, 2006-2008. Once a month I would help organize fun activities in a safe environment for about 50 students.

Secretary of *Beta Beta Beta*, a national biological honor society, Cedar Crest College, 2007-2008.

Member and Vice President of Black Student Union, Cedar Crest College, 2004-2008. Through my time as a member and one year tenure as vice president I helped raise awareness and contribute to diversity on a campus with a very small minority population.

TEACHING EXPERIENCE:

Advanced Topics in Human Genetics Teaching Assistant, Johns Hopkins University School of Medicine, 2010. Led 7-10 first year graduate students in weekly discussions on various topics related to human genetics, provided students with academic help, and assisted instructors.

Biology 231 (Genetics) Instructional Assistant and Tutor, Cedar Crest College, 2006-2008**.** Provided undergraduate students with academic help, study strategies and encouragement

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PUBLICATIONS:

Currier, D., Polk, R., and Roger H. Reeves. 2012. A Sonic hedgehog (Shh) response deficit in trisomic cells may be a common denominator for multiple features of Down syndrome, Progress in Brain Research 197:223-36.

ABSTRACTS:

Polk, Renita. (2012). Identification of Genetic Modifiers of Congenital Heart Defects. Poster presented at the Weinstein Cardiovascular Development Conference.

Polk, Renita. (2011). Identification of Genetic Modifiers of Congenital Heart Defects. Poster presented at the National Heart, Lung, and Blood Institute "*Genomics: Gene Discovery and Clinical Applications for Cardiovascular, Lung, and Blood Diseases" symposium*.

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